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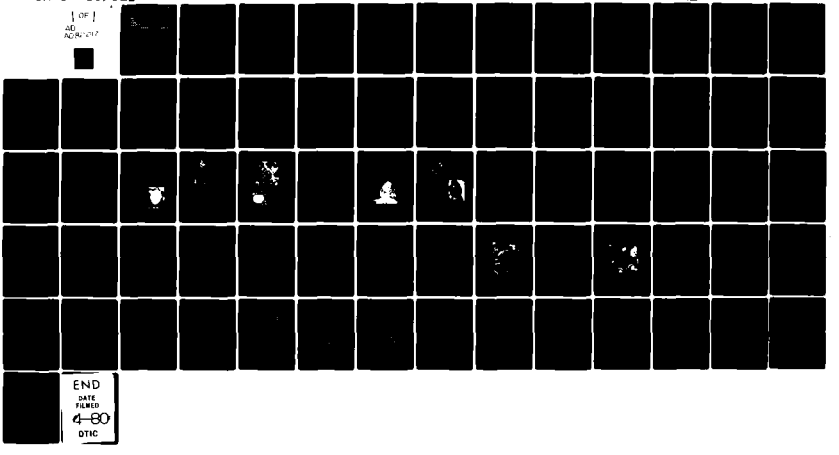

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ISOLATION AND CHARACTERIZATION OF PLASMODIAL AND BABESIAL ANTIGENS

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For the Period
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The objective of this project was the isolation and characterization of antigens of plasmodia and babesia. Part II is a critical review of procedures for the isolation of malarial parasites and for their fractionation. This review provides an analysis of the problems confronting those attempting to isolate antigenic materials from plasmodia. Parts III and IV are descriptions of research on the mechanisms by which the host resists plasmodial infection

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and by which the plasmodium defends itself from the host's response. These studies show clearly the importance of the merozoite capsule in the resistance of the blood inhabiting stages of plasmodia to the immune responses of the host. They also define the role of antibody and macrophages in the host's response to plasmodial infection. The final section, Part IV of this report, describes the isolation and characterization of a soluble component of Plasmodium berghei which induces immunity to challenge in rats. This component is obtained by gentle washing of free parasites and may be capsular material. It is proteinaceous and was shown by disk gel electrophoresis to contain only a small subset (2-4) of the proteins present in the parasites. Quantitative immmization studies indicate that on the basis of protein concentration this soluble material is about 20-fold more potent as an immunogen than are the unfractionated parasites. A similar purefied fraction of plasmodia of human origin, if it can be obtained, may be a candidate for use as a vaccine against malaria.

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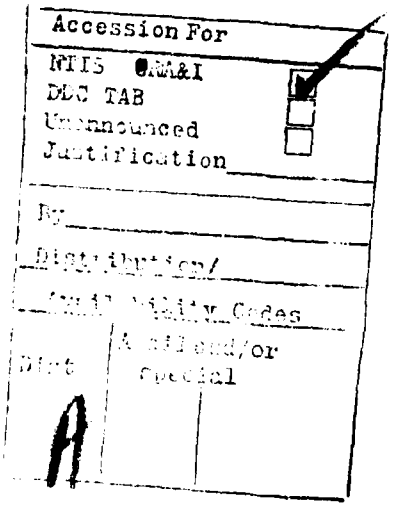

Bulletin of the World Health Organization, 55 (2-3): 317-331 (1977)

The isolation and fractionation of malaria-infected cells *

JULIUS P. KREIER [1]

This paper is a critical review of procedures for the isolation of malarial parasites from host cells and their fractionation. The procedures are grouped according to the stage of parasite being isolated, and the procedures for isolation of the erythrocytic stages are further grouped by techniques used. Some types of procedure are described for isolation of all stages of the parasite, both those in the invertebrate and vertebrate hosts. The uses and limitations of the various procedures are described. It is concluded that all the procedures are useful for some purposes, but that from a morphological standpoint only natural release in culture and continuous flow oscillation provide large yields of intact erythrocytic parasites free of host cell membranes.

The malarial parasite is a complex eukaryotic organism. The parasite develops in two hosts, an invertebrate and a vertebrate, and in those hosts it exists in a variety of morphologically distinct forms. In each host a variety of organ and cell systems are parasitized. Some of the stages exist only intracellularly, whereas others such as sporozoites and merozoites are specialized for passage from one host, or host cell, to another. The morphologist describes the organism in terms of plasma membranes, pellicule complexes, microtubules, nuclei, ribosomes, and a multitude of other structures; the biochemist talks about proteins, lipids, and carbohydrates; the physiologist discusses enzymes, and the immunologists and serologists are concerned with antigens. They are all, of course, talking about the components of the same parasite; only their terminology is different and reflects their training and interests. They are all confronted with the same problem, that of obtaining sufficient quantities of the parasite in forms suitable for their purposes. The problem of obtaining sufficient quantities of the parasite for study is complicated by the fact that the parasite has a complex life cycle. While all stages of the parasite almost certainly share common components (*79*), each stage also has unique components characteristic of the stage. Thus, even if it were possible to eliminate the problems in analysis caused by the parasite's intimate association with its host, it still remains necessary to separate the parasites by stage of development, and to separate the components of each stage of the parasite to determine which are common to all stages and which are unique. As some stages of the parasite may undergo antigenic variation (*17*), another level of complication is added to the analysis of the components of the parasite.

In this review I will attempt to summarize the literature which describes attempts by various people to isolate malaria parasites and to fractionate them into their component parts for study. A number of reviews and reports are available that contain sections on isolation and fractionation of malaria parasites and provide information on the implications of isolation and fractionation work (*164*, *161*, *13*, *107*, *162*, *89*, *49*, *50*, *155*, *1*).[a]

ISOLATION AND FRACTIONATION OF STAGES OF THE PARASITE FROM THE VERTEBRATE HOST

Exoerythrocytic stages including exoerythrocytic merozoites

The mass *in vitro* cultivation of exoerythrocytic stages of avian malaria parasites in cell monolayers

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[a] See also National Academy of Sciences. *Workshop on problems related to the development of an antimalarial vaccine*. National Research Council, Washington, DC, 1974 (mimeographed document).

is technically feasible (*39*, *68*). Exoerythrocytic merozoites may be obtained by centrifugation of the cell culture medium overlay at 4000 *g* for 30 min (*64*). Immunization of turkeys with formalin-killed exoerythrocytic merozoites prepared from such cultures conferred some stage-specific protection against exoerythrocytic forms of the parasite, but did not protect against infection by blood forms (*64*). Some evidence exists that injection of the avian merozoites will confer protection against infection with *P. berghei* sporozoites (*65*).

Plasmodium berghei yoelii exoerythrocytic schizonts stain well with fluorescent-labelled serum raised to blood stages of the parasite (*48*), indicating either that the fluorescent antibody test was not measuring response to antigens important in stimulating immunity or that, in rodent malaria, antigens stimulating protection are shared by exoerythrocytic and erythrocytic stages. The *in vitro* cultivation of exoerythrocytic stages of mammalian plasmodia, a prerequisite for studies of the antigenic constituents of this stage of the parasite, is very difficult. This difficulty arises partly from the fact that exoerythrocytic stages of mammalian plasmodia, unlike those of avian plasmodia, undergo only a limited number of generations of reproduction and must, therefore, always be produced from sporozoites, and partly because their host cell, the liver parenchyma cell, is difficult to cultivate. It is difficult to obtain sterile sporozoites from conventionally reared mosquitos, and this also complicates the culture procedure (*67*). Avian exoerythrocytic parasites will grow readily in mammalian liver cells (*8*), but only sparse growth of mammalian parasites in such cells has been reported (*44*).

Erythrocytic stages

General considerations. There is a very extensive literature on procedures for obtaining plasmodia from infected red cells. By some approaches, the infected red cells and the contained parasites are disrupted and then various biochemical or biophysical techniques are used to obtain the desired parasite components from the soluble mixture; by other approaches, an attempt is made to free morphologically intact parasites from the red cells and then to separate these parasites from the erythrocyte debris before solubilization and fractionation of the parasites is undertaken. Practically all of the procedures used for obtaining plasmodial components from red cells are variations of a few distinct techniques; these are hypotonic lysis, lysis by freezing and thawing, lysis with agents such as saponin, NH_4Cl, or antiserum and complement, lysis by sudden decreases in pressure, lysis by ultrasound, and the most recent procedure, cultivation to permit the parasites to mature and be released spontaneously. The first two of these procedures provide a soluble mixture of parasite and host components which must then be fractionated; the remainder of the procedures, with varying degrees of success, provide parasites freed from the host cells for further study.

Preparation of parasitized erythrocytes. The most economical and convenient source of erythrocytes infected with plasmodia for laboratory study is the blood of rodents infected with one of the various rodent plasmodia. The skills and knowledge needed for isolation and fractionation of plasmodia can easily be obtained using rodent material. Avian plasmodia are less suitable for study of isolation and fractionation of plasmodia, as the presence of the erythrocyte nucleus and its contained DNA complicate separation procedures. Parasitized blood cells can also be obtained from monkeys infected with their respective malarias if desired (*24*). The demonstration that *Plasmodium vivax* (*99*) and *Plasmodium falciparum* (*54*) grow well in *Aotus trivirgatus*, and the more recent successful *in vitro* culture of *Plasmodium falciparum* in erythrocytes (*147*, *62*), provide sources, other than infected humans and chimpanzees, of erythrocytes containing human plasmodia for study. Culture of erythrocytic stages of plasmodia in cells other than erythrocytes (*132*) is not yet a practical procedure.

Blood from animals or humans infected with plasmodia may be subjected to some preliminary treatments to remove unwanted components and to increase the proportion of infected erythrocytes before separation of the parasites from the host cells is undertaken. A first step is washing in physiological saline to remove plasma. If, during the washing steps, the buffy coat is removed along with the diluted plasma, the numbers of platelets and leucocytes will also be reduced. A more complete removal of leucocytes can be accomplished by passage of the resuspended, washed blood cells through a column of filter paper powder (*53*). Blood diluted 1: 6 in an appropriate medium may be passed directly through a column of filter paper powder (*104*). The column of powder should be wet with saline solution before use. The ability of the columns to retain leucocytes is limited. They can be overloaded, and then the

excess leucocytes will pass through. Forcing passage by pressure, or by excessive rinsing, to increase the speed of the process or to decrease the loss of red cells will dislodge leucocytes from the column. Attempts have also been made to remove leucocytes from infected blood by gradient techniques. The range of densities of leucocytes (*160*) overlaps that of parasitized red cells and thus these procedures are not very good. Of the various procedures reported for the removal of leucocytes from infected blood, the filter paper powder column technique, when used properly, is the best (*66*). Platelets may be removed by passage of the blood through a glass bead column (*114*).

Before one attempts to separate the parasite from the host red cell, it may be desirable to separate the parasitized red cells from the uninfected ones, or to attempt the separation of parasitized red cells by stage of development. Plasmodia are less dense than red cells. The more parasites a red cell contains, and the larger the parasites it contains, the less dense the host cell–parasite complex. Some plasmodia cause the red cell to enlarge, decreasing its density, and some plasmodia preferentially infect young erythrocytes which are less dense than mature erythrocytes. All these factors affect the isolation of parasitized erythrocytes by gradient techniques (*93*).

Eaton (*46*) collected schizont-containing rhesus monkey erythrocytes by allowing infected blood to settle slowly. The schizont-containing cells were present in increased concentration, just under the buffy coat. Gradients for separation may be made from sucrose (*156*), human albumin (*52*), bovine albumin (*111*), or phthalate (*93*). In very recent studies, Lund & Powers (*82*) concentrated schizont-containing *P. knowlesi*-infected erythrocytes on a discontinuous Ficoll gradient. Many schizont-containing cells were in a layer at the interface of the 20 and 25% Ficoll bands. McAlister & Gordon (*88*) separated *P. berghei*-infected cells by stage of development on a discontinuous Stracten II gradient. Schizont-containing cells had a specific gravity of less than 1.043, trophozoite-containing ones between 1.081 and 1.091, and uninfected erythrocytes and erythrocytes containing very small parasites had specific gravities greater than 1.091. Sucrose is an undesirable substance for gradient preparation because it is osmotically active. All reported studies have been done with parasitized erythrocytes, none with free parasites, although some of the workers do not make the distinction in their discussions.

For those working with a synchronized infection, parasites primarily in a given stage of development may be obtained by judicious choice of the time of blood collection. This system was used by Trager et al. (*148*) to obtain a population of *P. lophurae* trophozoites. If, however, the parasites are, as *P. berghei*, not naturally synchronized or lose their synchrony in culture, then one must either use techniques such as the gradient centrifugation procedures just described to obtain parasites in a given stage, or attempt to induce synchrony. Arnold and his associates (*5*, *125*) demonstrated that some degree of synchronization of *P. berghei* in mice could be induced by control of the photoperiod to which the host mice were exposed. Successful synchronization of *P. berghei* infection would facilitate isolation of specific stages of *P. berghei* from the blood, and greatly simplify the analysis of the developmental events in this otherwise most easily studied malaria parasite.

Hypotonic lysis. Some of the earliest successful attempts to prepare plasmodial antigens for serodiagnosis used hypotonic lysis of parasitized erythrocytes to release antigens. Coggeshall & Eaton (*25*) prepared a group-specific complement-fixing antigen from *P. knowlesi*-infected rhesus monkey erythrocytes by treating infected erythrocytes, probably at 37°C, for 48 hours with three times their volume of distilled water. After centrifugation, the haemoglobin-containing supernatant fluid was made isotonic by addition of NaCl. Later, the procedure was modified (*45*). The haemoglobin-containing fluid, produced by the distilled water lysis, was discarded and the solid material, containing a mixture of erythrocytic stroma and parasites in various stages of disruption, was collected. This material was then dried under vacuum and stored until needed. When it was to be used, it was ground, saline solution added, and then freeze–thawed three times. After centrifugation, the clear supernatant fluid was used for antigen in complement fixation tests. The material had the same type of group-specificity as that in the lysate used by Coggeshall & Eaton (*25*), but had the advantage of being relatively free of haemoglobin. Davis (*39*) separated the antigen from the haemoglobin in the crude lysate by precipitation of the antigen from the lysate. He did this by adding 35 g of $(NH_4)_2SO_4$ to each 100 ml of lysate.

Antigens prepared by procedures very similar to those of Dulaney & Stratman-Thomas (*45*) have been used by Mayer & Heidelberger (*86*) in studies

of the CF test in the diagnosis of malaria; by Stein & Desowitz (*138*) to sensitize red cells for use in a passive haemagglutination test; and by Chavin (*21*) as starting material for an analysis of parasite constituents.

Lysis by freezing and thawing. The freezing and thawing of the infected erythrocytes causes haemolysis and also damages the parasite's membrane. Following centrifugation to remove the membraneous structures, one obtains a deep red fluid which contains, in addition to haemoglobin, a variety of soluble products from the parasite and the host cell. The soluble parasite products obtained are commonly used in serodiagnostic tests. In the procedure used by Sadun & Gore (*112*), the infected cells are washed to remove the plasma, then a 50% suspension of the washed cells is prepared in saline and subjected to three cycles of freezing and thawing. The crude haemolysate is centrifuged at 2700 *g* for 30 min to remove insoluble components, and then may be stored frozen at −70°C until used. Sadun & Gore (*112*) fractionated the lysate by sequential elution from a Sephadex A-25 column with phosphate buffers of increasing concentrations. Haemoglobin was dislodged from the column by a 0.04 mol/litre buffer, pH 7.5, while a parasite fraction that was dislodged by a 0.1 mol/litre buffer, pH 6.5, proved to be excellent as a diagnostic antigen in the soluble antigen fluorescent antibody test. No attempt was made to determine what the antigen was, but Sadun & Gore (*112*) suggested that lysis of the host cell–parasite complex should conserve soluble parasite products that would be lost by procedures which lysed the erythrocyte to remove haemoglobin before the parasite was extracted. McAlister (*87*) examined the insoluble material left after freeze–thawing by a procedure similar to that of Sadun & Gore (*112*). He found some unlysed erythrocytes, many morphologically intact parasites, particularly trophozoites, and much stromal material. McAlister (*87*) found that the serologically active materials could be precipitated from the haemoglobin-containing solution by adding $(NH_4)_2SO_4$, and that the precipitated material, after solution, could be further purified by passage through a G-100 Sephadex column. The antigenically active material obtained from the column was in the void volume and had a molecular weight of at least 800 000. McAlister (*87*) confirmed that the antigen was useful in a soluble antigen fluorescent antibody test and also showed its usefulness in a passive haemagglutination test. Mathews et al. (*85*) used antigen released by freeze–thawing in passive haemagglutination tests for malaria. The antigen they routinely use for sensitizing the carrier erythrocytes is a crude lysate of parasitized erythrocytes obtained by freezing and then thawing the washed infected erythrocytes, passing the crude lysate through a Ribe Cell Fractionator at a pressure of 117.2 MPa, and then centrifuging the lysate at 12 000 *g* for 10 min. The selective absorption of the serologically active antigens on to the tanned red cells is the only additional fractionation used. A variant of this type of procedure subjects the freeze-thawed parasitized erythrocytes to a 10-second burst of sonic energy, instead of passing it through a Ribe Fractionator before it is used to sensitize the red cells (*91*). Unfractionated freeze–thawed *P. knowlesi* schizont-containing erythrocytes have been used as antigen in vaccination studies (*18*). The materials appear to stimulate a moderate degree of immunity to challenge infection.

Lysis with saponin. The use of saponin to lyse erythrocytes, and centrifugation to collect the unlysed parasites, was introduced by Christopher & Fulton (*22*). The procedure, which has been changed little by subsequent workers, is widely used today as the first step in reducing the proportion of host material in parasite preparations. Stauber & Walker (*136*) recognized that parasites prepared by saponin lysis of parasitized erythrocytes were, with rare exceptions, contained within the collapsed erythrocyte membranes. They recognized this because the avian parasites with which they worked remained bound to the host cell nucleus after lysis of the erythrocyte. The erythrocyte membranes surrounding the parasites cannot be resolved by light microscopy of Giemsa-stained films of the parasites, and it is owing to this fact that the mistaken view that saponin lysis yields free parasites arose. Phase-contrast microscopy of wet preparations, or electron microscopic examination of thin sections of the parasites (*2*, *28*, *100*, *101*, *102*), is required to demonstrate the erythrocyte membrane envelope around the parasites.

Dr A. Zuckerman has been a major force in the reintroduction and popularization of the saponin lysis technique (*134*) and her adaptation of the original procedure has been described in detail (*165*). In brief, Zuckerman's procedure is as follows. The blood is collected in a suitable anticoagulant solution, for example, sodium citrate. The plasma is removed following centrifugation and the erythrocytes are washed in phosphate-buffered saline by a

series of centrifugations. Leucocytes and platelets may be removed in part with the plasma if the buffy coat is also removed. The blood cells suspended in buffered saline may be further purified of leucocytes and platelets by sedimentation through a 3.6% dextran solution. The erythrocytes clump and sediment rapidly; the leucocytes are discarded with the supernatant fluid.

The sedimented cells are washed in phosphate-buffered saline and then 5 times their volume of a solution that contains 1 part of saponin in 7500 parts of saline at 37°C is added. The mixture is incubated at 37°C for 15 min with occasional stirring. The suspension is then chilled and centrifuged for 30 seconds at 10 000 rev/min. The supernatant fluid is discarded and the procedure repeated, the only difference being that incubation in saponin is only for 10 min, not 15. The parasites and erythrocyte membranes are in the pellet after the last centrifugation, and are ready for use after washing in saline to reduce the saponin contamination.

Dr Zuckerman recognized the importance of eliminating leucocytes from the parasitized erythrocytes before lysis; however, the dextran procedure she recommends, as mentioned earlier (page 319), is not as good as the filter paper column technique for removing leucocytes.

Since it has been recognized that the parasites prepared by saponin lysis are contained in the erythrocyte membrane, attempts have been made to remove the membrane. Stauber & Walker (*136*) used enzymatic digestion. The digested parasite preparations, unlike the undigested preparations, could be agglutinated by immune serum, but were not free of membranes (*149*). Kreier et al. (*77*) fixed saponin-prepared avian parasites with formalin and then separated the parasites from the erythrocyte nuclei by sonication. These free parasites were morphologically intact and could be agglutinated with immune serum but were not suitable for solubilization and further analysis because of the fixation. Cook et al. (*28*) attempted to remove the erythrocyte membranes from *P. knowlesi* prepared by saponin lysis by a variety of techniques. They observed that fixation with glutaraldehyde followed by mechanical agitation in a Waring blender would yield morphologically intact, free parasites, but they observed that these fixed parasites were of little use for subsequent studies. They also found that mechanical agitation without prior fixation disrupted the parasites, and that treatment with sodium dodecyl sulfate lysed the parasites.

Most scientists using the saponin-prepared parasites have accepted the presence of the erythrocyte membranes in the preparations, and have tried to evaluate their importance by comparative study of saponin-prepared erythrocyte membranes.

For most studies of the parasites, the collection of the morphologically intact parasites after lysis of the host erythrocytes with saponin, is only the first step in the process. The second step is generally the disruption of the collected parasites and the separation of the soluble from the insoluble material by centrifugation. A great variety of procedures have been used to disrupt the parasites. Sherman & Hull (*124*) obtained proteins and haemozoin from *P. lophurae* for biochemical analysis by alternate freezing and thawing, and Sherman (*123*) used freeze–thawed parasites for antigenic analysis. Cook et al. (*29*) obtained ribosomes from *P. knowlesi* after disruption of the saponin-prepared parasites by distilled water lysis. Zuckerman introduced the use of the Hughes press (*134*) to disrupt the parasites and carried out analysis of the soluble components by disc gel immunodiffusion and immunization techniques (*135*, *60*, *61*). A variety of techniques have been used for grinding the parasites. Jerusalem (*70*, *72*) used a Potter Homogenizer to grind the parasites. Corradetti et al. (*30*) first filtered the saponin-lysed material, then ground the parasites obtained in the filtrate in a Virtis blender with quartz powder, and Rock et al. (*105*) ground the parasites in an all-glass Ten Broeck homogenizer.

A variety of individuals have used ultrasound either alone or with other procedures to disrupt the parasites (*43*, *131*, *153*, *83*). In some cases the parasites were lyophilized before grinding (*12*, *159*).

Lysis with ammonium chloride. Plasmodia may be freed from erythrocytes by treatment of the infected erythrocytes with a 0.155 mol/litre NH_4Cl solution buffered to pH 7.4 with 0.17 mol/litre Tris buffer. One volume of a 50% erythrocyte suspension is added to 9 volumes of the 37°C buffered NH_4Cl solution and the mixture incubated at 37°C for 3 min. Subsequently, the mixture is centrifuged at 500 *g* for 12 min at room temperature and the sediment is washed twice in a MEM solution with 10% fetal calf serum. The parasites in the sediment are reported to be free of red cell membrane, morphologically intact, and suitable for antigenic studies (*84*). The crucial importance of controlling the time of exposure of the parasites to the lytic solution, if the parasites are not also to be lysed,

was emphasized in a later study of the technique (*103*).

Lysis with antiserum and complement. The release of plasmodia from host erythrocytes by use of specific anti-host erythrocyte antiserum and complement has been used most commonly by individuals culturing free parasites (*145*, *146*), and by individuals studying plasmodial metabolism and enzyme activity (*11*, *80*, *150*). A fairly typical procedure is that of Trager et al. (*148*). Washed infected erythrocytes are suspended in four times their volume of a balanced saline solution containing a lysed erythrocyte extract. To 6.3 ml of the suspension, 0.13 ml of guinea-pig serum and 0.7 ml of rabbit origin anti-erythrocyte serum are added. The mixture is incubated for 30 min at 40°C on a rocking platform, and is swirled vigorously after 15 min and again at the end of the 30-min incubation period. The material is then sucked in and out of a pipette and transferred to a centrifuge tube and centrifuged just long enough to result in clearing of the fluid. The freed parasites are in the supernatant. Earlier versions of the procedure (*145*) included enzyme digestions with trypsin and deoxyribonuclease, but these preparations had lowered viability (*148*). Some procedures passed the supernatants obtained from the centrifuged lysate through a 5-μm filter at a pressure of 82.7 kPa (*15*).

Langreth & Trager (*81*) presented electronmicroscopic evidence that *P. lophurae* trophozoites prepared by immune lysis were free of host membranes, and sufficiently undamaged that they would develop into schizonts extracellularly in culture. An interesting variant of the immune lysis procedure was that of Walter (*152*), who brought about *in vivo* release of *P. berghei* parasites by immunizing mice with normal rat erythrocytes and then infecting the mice with *P. berghei* parasitized rat erythrocytes. As the resulting infections were synchronized for several generations, Walter (*152*) concluded that only the merozoites released by the *in vivo* haemolysis were infective. This method of achieving synchrony, if repeatable, would facilitate evaluation of the components of the intraerythrocytic development stages of the normally unsynchronized *P. berghei*.

Lysis by sudden decreases in pressure. The technique for release of plasmodia from erythrocytes by decompression under the controlled conditions given by a French pressure cell, was developed by D'Antonio (*37*). A 20% suspension of washed infected erythrocytes is prepared in a buffered saline solution. This is extruded from the orifice of the French pressure cell at an appropriate pressure, usually between 5.5 and 6.9 MPa. The pressure chosen depends on the parasite–host cell system being studied, and must be selected on the basis of trial and error (*34*). The effluent from the cell is centrifuged at 50 *g* for 10 min to remove the unbroken red cells and gross debris, and then the parasites are collected by centrifugation of the supernatant at 3500 *g* for 5 min. The parasites are washed, then suspended in 7 volumes of saline and disrupted by passage through the French pressure cell at 124–138 MPa. The effluent is cleared by centrifugation at 10 000 *g* for 30 min (*37*). The supernatant fluid from this centrifugation was used in a complement fixation test (*38*). On passage through a G-200 Sephadex gel column the material in the void volume proved to contain most of the CF activity. The CF antigen is stable for 2 months at 4°C and may be lyophilized without loss of activity if 1% polyvinylpyrrolidone is added (*37*).

Parasites prepared by the French pressure cell technique have been used to study the enzymes of *P. knowlesi* (*114*). On fractionation these parasites have yielded a lytic factor of lipid nature which lyses normal erythrocytes (*51*). The most extensive use of French pressure cell released plasmodia and their fractions has been in immunization experiments in Dr Silverman's laboratory (*35*, *36*, *127*, *115*, *116*). Speer et al. (*133*) reported that the immunizing material in the void volume of G-200 Sephadex column fractionated, French pressure cell released, and disrupted plasmodia was largely vesicles of parasite membrane origin.

Chow & Kreier (*23*) obtained free parasites by the very simple technique of extruding a 10% suspension of washed *P. berghei*-infected red cells through a 27-gauge needle by hand pressure on the syringe plunger. The approximate rate of extrusion was 0.5 ml/s. The effluent was diluted four-fold, and then centrifuged briefly at low gravity force to remove unbroken red cells; the supernatant was then centrifuged at 3500 *g* for 8 min to collect the parasites. This very simple procedure, within the reach of almost any laboratory, yielded parasites suitable for studies of the action of phagocytes on free parasites and parasitized erythrocytes.

Lysis by ultrasound. It was shown in 1956 (*151*), that ultrasound would disrupt red cells and release plasmodia. Later, it was shown that parasite yield increased only to a limited degree with increases in the intensity of the sonic energy used, and the

duration of the exposure (*108*), and that while low frequency ultrasound waves appeared to disrupt red cells more efficiently than high frequency waves, no frequency existed that would break red cells but not plasmodia (*102*). It is thus apparent that in batch sonication systems, no combination of time, intensity, and frequency can be selected that will break red cells without also subsequently breaking the released parasites. Thus, the problem in the use of sonic energy for release of plasmodia was one of developing a system to permit red cell disruption, and then achieve prompt removal of the freed parasites from the sound field. This problem was resolved by the development of a continuous flow system (*100*). The crucial technical aspect of the system is the design of the chamber. It must have a small void volume. The washed parasitized red cells must pass through the ultrasound field in an ordered fashion, and there must be no eddies in which parasitized erythrocytes and parasites may be trapped and subjected to prolonged exposure to the disruptive forces of the ultrasound. The optimum rate of flow for maximum yield of parasites must be selected by experimentation for each continuous flow system. Almost any reasonably powerful commercial sonicator may be used if fitted with a chamber that permits the ordered passage of a thin layer of suspended parasitized cells through the sound field. A satisfactory system has been described in detail (*100*). Leucocytes and platelets must be removed from the blood cells by a suitable procedure before sonication (page 318), for sonication will cause release of nucleic acids which entrap the parasites and prevent subsequent separation of the parasites from the erythrocyte debris. Prior & Kreier (*100, 101*) separated the freed parasites from unbroken erythrocytes and erythrocyte debris by differential centrifugation. A short, low-force centrifugation is used to remove the unbroken red cells, then a longer, higher-force centrifugation pellets the parasites, and the finely divided debris remains in the supernatant fluid. A practical procedure to select the conditions of centrifugation required to obtain parasites is to prepare a series of identical tubes of the effluent from the sonication chamber, then choose an arbitrary gravity force, for example 300 *g*, and centrifuge for 5, 10, 15, 20, 25, and 30 minutes. Examination of the pellets will permit choice of appropriate centrifugation times at the selected gravity force to obtain a clean parasite preparation. Higher gravity forces may be used with shorter times. Alternatively, the supernatant fluid from a single sample may be centrifuged several times at a constant gravity force and each pellet examined to determine the appropriate times for centrifugation to obtain clean parasites.

Thin-section electron micrographs have been published showing that relatively undamaged parasites, free of entrapping erythrocyte membranes, may be prepared by the sonication technique (*100, 101, 76*). Parasites prepared by continuous-flow sonication have been shown to be suitable as sources of antigen for complement fixation tests (*100, 101*), for studies of the mode of action of the host against the parasite (*57, 58, 59*), and in vaccination studies (*113*). The excellent state of preservation of the sonically freed parasites has made them very useful for the study of the surface properties of free parasites, an area not previously amenable to study (*117, 119, 121*). The technique is not limited to use with plasmodia; *Babesia* have been freed by essentially the same procedures (*55, 75*).

Natural release. All of the procedures for obtaining erythrocytic stages of plasmodia described so far in this review have involved the use of chemical or physical forces to disrupt the host erythrocyte membrane to release the contained parasites, or to release components of the parasites. The parasites obtained are in whatever stage of development they were in at the time of erythrocyte rupture. Mitchell et al. (*94*) were able to culture erythrocytes containing *P. knowlesi* schizonts long enough for these schizonts to mature and release merozoites which were then collected by differential centrifugation. Later, Dennis et al. (*41*) improved the procedure by culturing the schizonts in a chamber, the floor of which was a 2-μm pore size polycarbonate sieve through which the merozoites were drawn as they were released. More recently, *in vitro* techniques for short-term culture (*126*) and finally serial culture of *P. falciparum* (*147, 62*) have been developed. Culture of *P. falciparum* in a chamber like that used by Dennis et al. (*41*) with *P. knowlesi* will permit the collection of *P. falciparum* merozoites in quantity. Culture techniques for other plasmodia, *P. berghei* (*129*) for example, are less advanced. Despite the newness of the natural release techniques for obtaining erythrocytic merozoites, the usefulness of the culture procedures for obtaining merozoites for study of host–parasite interactions (*26, 92*) is proved. The most significant use of the merozoites obtained by natural release has been in vaccination studies (*32, 95, 96*).

Comparison of procedures. When one is evaluating procedures for separating plasmodia from host cells, or for obtaining plasmodial components, one must realize that the value of the procedures is to a large degree determined by the objectives of the individual making the study. In general, the simplest procedure that provides parasite components in a form satisfactory for the studies being done is the best procedure. Thus Kagan and his associates have found an admittedly crude lysate of infected red cells satisfactory for sensitizing tanned red cells for use in seriodiagnosis (*85*), and Sadun & Gore (*112*) used a freeze–thawed lysate of whole infected red cells as the starting material for fractionation to obtain an antigen for a soluble antigen fluorescent antibody test. These workers considered that the materials present in the red cell cytoplasm would be lost by preliminary freeing of the parasites from the host cells. Lund & Powers (*82*) found that for a passive haemagglutination test the antigen obtained by freeze–thawing schizont-infected red cells was as good as the antigen obtained from saponin-prepared parasites, but the yield from the free parasites was smaller, indicating that antigen may be lost with the haemoglobin as suggested by Sadun & Gore (*112*). D'Antonio et al. (*37*) compared the value, in diagnostic CF tests, of antigens prepared by French pressure cell lysis of infected erythrocytes to ones prepared by the older distilled water lysis techniques. The French pressure cell antigens were less anticomplementary and more potent than were those prepared by distilled water lysis. Prior & Kreier (*101*) found that sonically freed parasites provided excellent CF antigens, but they did not make direct comparisons with other antigens. No systematic evaluation of plasmodial antigens for use in serology appears to have been undertaken. The techniques for systematic evaluation of antigens for specificity and potency have been described in detail (*49*, *50*) and need only be applied systematically.

Comparative evaluation of various antigen preparations for immunization is an undeveloped field. Jerusalem & Eling (*71*) made a comparison of the immunogenicity of saponin-released, haemolytic antiserum and complement-released, and dilute formalin-released *P. berghei* as immunogens in mice. They concluded that the method of release of the parasite was not related to the antigenicity. Desowitz (*42*) compared the immunogenicity of several subfractions of saponin-prepared *P. berghei* parasites in rats in what was basically a study of adjuvants. He reported that alum-precipitated components of the soluble portions of saponin-prepared *P. berghei* parasites were not immunogenic. Saul & Kreier (*113*) compared the immunogenicity of various fractions of sonically-lysed *P. berghei* in rats. The techniques they describe could be applied to a comparison of the various antigens currently being used. These workers emphasize the importance of a careful quantitative analysis of the immunogens if valid comparisons are to be made. Most proposed vaccines have not been tested against other proposed vaccines in any systematic way.

Since many claims have been made that the procedures for preparing plasmodia would yield morphologically intact parasites free of host membranes, it was perhaps inevitable that electron microscope techniques would be used to evaluate the claims. Bahr (*6*) concluded that no procedure available at the time he made his study yielded morphologically intact parasites free of host cell membranes. Kilby & Silverman (*73*) made a fairly careful comparison of *P. berghei* released by the French pressure cell, saponin, antiserum and complement, and distilled water techniques and they concluded that lysis by distilled water disrupted the parasites but did not remove host membranes; saponin yielded parasites still in host membranes; and antiserum and complement did not free many parasites. They reported that the French pressure technique released the largest proportion of structurally intact parasites generally lacking closely associated host cell membranes. Cook et al. (*28*) and Cook & Aikawa (*27*) disputed Kilby & Silvermans' conclusions about the French pressure cell technique, as did Trager et al. (*148*). These individuals provided sound evidence that the French pressure cell procedure frees few parasites from host membranes, and disrupts many of those that are freed. Cook et al. (*28*) considered that the saponin procedure provided the largest yield of morphologically intact parasites, but observed that these are in host cell membrane ghosts. Aikawa & Cook (*2*) confirmed that saponin does not remove host cell membrane from around parasites and that the French pressure cell produces a disrupted mixture of materials. Aikawa & Cook (*2*) emphasized that morphological evaluation requires low power electron microscope scans of fields of parasites to be of any value. They noted that all procedures will provide some free parasites.

The two procedures that actually provide good

yields of morphologically intact free erythrocytic parasites, i.e., continuous-flow sonic oscillation and natural release in culture, did not exist at the time the comparisons just discussed were made. Adequate electron microscopic evidence of the efficacy of these two procedures for freeing parasites exists (*100, 101, 103, 76, 41*). The natural-release procedure yields merozoites; the sonic procedure yields free parasites of normally intraerythrocytic types.

The identification of erythrocyte membrane material in disrupted parasite preparations has been a problem. Erythrocyte membranes will fix complement with sera of individuals with malaria (*63*), and the active components, which are at least in part lipids, will sensitize erythrocytes for haemagglutination and will precipitate in gel with serum from infected individuals (*120*). It has recently been shown that erythrocyte membranes can be differentiated from parasite membranes by their affinity for colloidal iron stains (*118*), or by their affinity for a variety of lectins (*121*). These newly developed staining procedures will make possible the resolution of the question of the origin of the membraneous profiles present in French pressure cell-prepared parasite preparations (*9, 36*). It will, of course, never be possible to eliminate host material completely from preparations of intraerythrocytic plasmodia because of the presence of food vacuoles in the parasites (*106*).

Soluble antigen in the plasma

Soluble plasmodial materials occur in the plasma of acutely infected monkeys (*47*), ducks (*143*), mice (*69*), chickens (*139*), and men (*90*). The material in the plasma of *P. knowlesi*-infected monkeys is active in CF tests, but does not stimulate protective immunity (*47*). Soluble parasite material in the plasma of mice infected with *P. berghei* (*69*) and chickens infected with *P. gallinaceum* (*139*) did stimulate protective immunity on injection into uninfected hosts. Cox et al. (*33*) considered the soluble parasite components in the serum to have a very broad antigenic specificity for intraerythrocytic parasites, but it is probable that autoantibodies to host lipids, which contaminated their preparations, were responsible for these results (*74, 120*). The soluble parasite components in chicken serum are proteinaceous. The soluble parasite components in the serum can be separated from the serum by precipitation with salts, by ultracentrifugation, and by a variety of column chromatography techniques (*140*). The parasite materials in the serum of chickens with *Plasmodium gallinaceum* have been used to sensitize latex for a tube latex agglutination test (*142*), and those in serum of rats will precipitate in gel with immune serum from rats (*163*). There are at least three soluble parasite components in the serum of infected chickens. These have molecular weights of 500 000–1 000 000, 150 000–250 000, and less than 70 000 (*130*). Soluble parasite products in the serum of humans with *Plasmodium-falciparum* infections have antigenic specificities similar to those of some of the components of the parasite body (*158, 159*). The soluble parasite components in the serum of *P. falciparum* infected humans are probably released at the time of rupture of schizonts and at the time of penetration of red cells by the merozoites (*157*).

Parasite-associated materials in the erythrocyte

Eaton (*46*) observed that immune serum from monkeys infected with *P. knowlesi* would agglutinate schizont-containing red cells. Uninfected erythrocytes from the blood were not agglutinated, nor were erythrocytes containing rings or more advanced trophozoites. Thus, these parasite materials must make their way into the red cell membrane from the inside, through the erythrocyte cytoplasm, as the parasites mature. The parasite materials in the membranes of erythrocytes containing *P. knowlesi* schizonts are variant antigens (*16*). Erythrocytes containing various other plasmodia have parasite associated materials in their cytoplasm (*3*), but as specific antiplasmodial antiserum will not usually agglutinate these erythrocytes (*14*), these materials probably do not extend through the membrane. Todorovic et al. (*141*) reported that fluorescein-labelled antibody specific for soluble antigens in the serum caused fluorescence and stimulated phagocytosis of erythrocytes of chickens with *P. gallinaceum* infection, and caused fluorescence of free merozoites. These results would suggest that the parasite-associated materials in or on the erythrocytes, and on the merozoites, and the soluble materials in the serum are antigenically at least similar. Unfortunately, this work was done with outbred chickens, and it is not possible to exclude the possibility that the results reported were caused by antibodies to blood group antigens rather than parasite antigens. Attempts to isolate the membrane-associated antigens for study do not appear to have been made.

ISOLATION OF STAGES FROM THE INVERTEBRATE

Gametocytes, oocysts, and other developmental stages

The recent report that immunization of chickens with formalin-treated or X-irradiated *P. gallinaceum*-infected red cells would inhibit oocyst formation in mosquitos that fed on the chickens, while barely affecting the asexual infections in the chickens (*56*), has suggested a possible new method of malaria control. Gwadz's (*56*) procedure of immunization used blood stage parasites, including gametocytes, to stimulate the production of antibodies, which when taken into the mosquito's gut with the blood meal presumably acted upon the gametes when they were released. Carter & Chen (*19*) obtained a higher degree of inhibition of oocyst formation by immunization with gametes. To obtain gametes, Carter & Chen (*19*) collected infected blood-containing gametocytes, washed the blood cells in a medium that inhibited gamete release, then suspended the gametocytes in a solution that stimulated gamete release. The gametes were collected by a series of centrifugations and the preparations were irradiated to suppress infectivity of contaminating asexual parasites. Gametocytes of *P. falciparum* can now be produced *in vitro* (*62, 128*), and this will possibly permit isolation of gametes of human plasmodia by techniques similar to those used by Carter & Chen (*19*).

Plasmodium berghei ookinetes may be isolated from mosquito midguts in large numbers (*154*). Midguts are dissected from mosquitos 9–12 or 18–24 hours after engorgement. A variety of antibiotics are added to the medium in which the midguts are suspended. The guts are ground by 5–10 strokes in a Ten Broeck tissue grinder and the homogenate digested with collagenase and hyaluronidase at 20–21°C for 45–60 min while being gently stirred. The digest is then centrifuged at 50 *g* for 5 min to remove gross debris, and the supernatant is saved. The pellet is resuspended and the process repeated a total of 5 times. The pooled supernatants are centrifuged at 500 *g* for 15 min to sediment the ookinetes. The ookinetes in the pellet are then further cleaned by gradient centrifugation. The ookinetes are morphologically intact and motile, but because of rather heavy contamination with microorganisms are not very suitable for culture. To obtain the mosquito stages of plasmodia uncontaminated with bacteria and yeasts, two courses of action may be followed; one course is to raise mosquitos free of living microorganisms (*144*), the other is to culture directly from the blood those stages that are normally in the mosquito (*20*). Mosquito cell cultures are commonly used as support for the cultured plasmodia. Sporozoites developed from oocysts in the presence of mosquito cells (*7*) and ookinetes developed from gametocytes in such systems (*4, 110*). Ookinetes will develop from gametocytes in cultures of fat head minnow epithelial cells also (*109*), but ookinete yields in cell culture systems are small, and further development does not occur (*122*).

Sporozoites

It is hoped some day, to obtain masses of sterile sporozoites from cultures inoculated with gametocyte-containing blood; this is not at present possible. The common sources of sporozoites for study are the salivary glands or whole bodies of infected mosquitos. A procedure for obtaining relatively small numbers of clean sporozoites involves dissecting the salivary glands from the mosquito, placing the freed glands under a coverslip in saline solution, and causing a current of fluid to pass under the coverslip by dropping saline on one side, and withdrawing it from the other with a capillary tube. The sporozoites leave the glands, enter the saline and are collected with it. Simple centrifugation permits concentration of the sporozoites in the saline (*31*). A procedure for obtaining relatively large numbers of sporozoites involves grinding whole mosquitos, or mosquito thoraxes or abdomens, suspended in tissue culture medium 199, with a loose fitting Teflon tissue grinder. After grinding, the heavier fragments are eliminated by centrifugation and then the sporozoites are separated from most of the remaining debris by centrifugation on a bovine serum albumin–Renografin gradient. The sporozoites are concentrated in the region of the gradient with a specific gravity of about 1.10 (*78*). Bosworth et al. (*10*) described a procedure for mass isolation of *Anopheles stephensi* salivary glands infected with sporozoites. Decapitated mosquitos are washed on a 22-gauge mesh plastic screen, then spread as a monolayer on a glass plate between tracks formed by feeler gauges of specific thicknesses which serve as spacers. A roller is passed over the tracks. The salivary glands are expelled from the bodies, but the bodies are not crushed because the spacers maintain an appropriate distance between the roller and the glass plate. The expelled glands may be washed from the bodies by screening, and collected for further processing to obtain sporozoites.

Inoculation with X-irradiated sporozoites induces immunity against sporozoite-induced infection, but attempts to isolate an immunogenic subfraction of the sporozoites have not succeeded (*98*).

CONCLUSIONS

Procedures are available for obtaining quantities of exoerythrocytic merozoites of the avian plasmodia, but we do not have satisfactory procedures for obtaining exoerythrocytic merozoites of mammalian plasmodia, nor do we have any procedures for obtaining the intercellular forms of exoerythrocytic stages of plasmodia.

A variety of procedures for obtaining intraerythrocytic plasmodia have been described. All have their uses and limitations. Continuous flow sonication will provide morphologically intact intraerythrocytic plasmodia free of host cell membranes, and spontaneous release of cultured schizonts provides erythrocytic merozoites. These two procedures together will provide free parasites of all the erythrocytic development stages. A variety of procedures are available for precipitating soluble products of the parasites from the plasma and from lysates of infected red cells, but little work has been done on the isolation of parasite-associated materials from the erythrocyte membrane. Recently developed techniques for culture of the erythrocytic stages of some species of plasmodia will make this stage easier to study. While procedures are available for obtaining preparations of many of the stages of plasmodia that occur in the mosquito, in particular gametes, ookinetes, and sporozoites, our inability to culture these stages for more than short periods remains a problem.

RÉSUMÉ

ISOLEMENT ET FRACTIONNEMENT DES CELLULES IMPALUDÉES

Ce document passe en revue les méthodes applicables à l'isolement des parasites du paludisme à partir des cellules hôtes, et à leur fractionnement. Les méthodes sont groupées en fonction du stade du parasite isolé; pour les cycles érythrocytaires, elles sont encore groupées par techniques. Certaines méthodes décrites concernent tous les stades parasitaires, tant chez les hôtes invertébrés que vertébrés. L'auteur indique les emplois et les limites des diverses méthodes et conclut que toutes ont leur utilité mais que, du point de vue morphologique, seules la production naturelle en culture et le traitement par ultrasons en continu fournissent de grandes quantités de parasites érythrocytaires intacts, exempts de membranes des cellules hôtes.

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Demonstration of the Role of Cytophilic Antibody in Resistance to Malaria Parasites (*Plasmodium berghei*) in Rats†

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This paper reports the results of a study of the nature of the immune response against *Plasmodium berghei* parasites by inbred rats. A macrophage-cytophilic antibody specific for malarial antigens was identified and characterized. Detection of the antibody on the macrophage surface was accomplished by the parasite adherence tests and by the indirect fluorescent antibody technique. Isolation and purification of the macrophage-cytophilic and opsonic antibodies from hyperimmune rat serum was accomplished by QAE-Sephadex A-50 elution chromatography, and of the macrophage-cytophilic antibody by adsorption with and elution from syngeneic macrophages as well. Characterization of the cytophilic antibody as immunoglobulin G_1 was done by immunoelectrophoresis and by Ouchterlony-type double diffusion in gel. Passive protection tests in weanling inbred rats have demonstrated that the opsonizing antibody conferred some protection against *P. berghei*. The macrophage-cytophilic antibody, on the other hand, was not protective alone but acted synergistically with the opsonizing antibody.

It is well established that antimalarial antibodies are capable of protecting rodents against malarial infections (9, 32). Antimalarial serum antibodies have been shown to coat free parasites, and the suggestion has been made that protection is achieved at the merozoite stage (7, 20). Previous work (13, 16, 20) has also shown that free parasites, coated with immune serum in vitro, remain capable of initiating infection. However, Hamburger and Kreier (13) have additionally demonstrated that such antibody-coated parasites do not retain full infectivity if they are accompanied into the test animal by immune serum. Chow and Kreier (6) concluded that immune serum enhances the phagocytic capability of both normal and immune macrophages.

Immunoglobulins associated with macrophages were first reported in 1954 by Girard and Murray (12); then, in 1963, Boyden (4) introduced a rosette-forming procedure utilizing macrophage monolayers to conveniently demonstrate the presence of such antibodies. The definition of cytophilic antibody proposed by Boyden (4) and by Sorkin (28) stated explicitly that the binding of antigen to cytophilic antibody takes place subsequent to cellular fixation of the antibody, with the consequent implication that these antibodies are capable of cellular fixation before their combination with antigen (31). This usage of the term "cytophilic antibody" has been accepted by the present authors and will be the definition of the term as used herein.

Both Parrish (23) and Tizard (30) have reported that opsonizing antibodies are not cytophilic before combination with specific antigen and therefore are not identical to those antibodies that are detected by Boyden's macrophage rosette system.

According to Tizard (31), the amount of cytophilic antibody found in the serum will depend upon both the avidity with which the antibodies bind to the macrophage receptors and the availability of those receptors. High binding avidity and ample receptor availability may result in undetectable levels of cytophilic antibody in the serum, with those antibodies that are found being of perhaps the lowest binding avidity for the macrophage receptors. For these reasons, we have chosen to use hyperimmunized rats for our source of cytophilic antibody in an effort to effect a spillover of relatively high-avidity cytophilic antibodies into the serum.

In the studies related here, we investigated the possibility that immune serum might directly arm macrophages against *P. berghei* parasites by the agency of a macrophage-cytophilic antibody, and we undertook to demonstrate by various procedures the presence of a macrophage-cytophilic antibody on nonimmune mac-

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rophages that had been presensitized in immune serum. In vitro experiments that demonstrated the capacity of a specific macrophage-cytophilic antibody to promote adherence of free parasites to macrophages were performed first. After this, an attempt was made to adsorb the cytophilic antibody component out of hyperimmune antiserum by using normal macrophages. The effectiveness of the adsorption was judged by the blockade of subsequent fluorescent staining of the macrophages by fluorescein-conjugated hyperimmune rat serum (HIRS) globulins that contained macrophage-cytophilic antibodies. Physical separation of the cytophilic antibodies from the opsonic antibodies in HIRS was attempted both by column chromatography and by adsorption-elution with macrophages. Boyden (4) and Sorkin (28) have reported that heating a macrophage suspension to 56°C for 30 min is sufficient to detach cytophilic antibodies. This process presumably destroys the receptor sites on the cell membranes, releasing the antibodies, which remain reactive. HIRS, serum fractions, and eluates were tested in vivo for protective effects.

MATERIALS AND METHODS

Malarial parasites. The *P. berghei* strain used in this study was obtained from M. Aikawa (Case Western Reserve University, Cleveland, Ohio) and originated from the Walter Reed Army Institute of Research. A pool of infected mouse blood in 10% glycerol, aliquoted and frozen in liquid nitrogen, served as the reference stable source for all infectious inocula used in this study. Thawed material was inoculated first in a mouse, then passed into a weanling rat, whose infected blood served as the infectious inocula for test animals and for animals harvested either for immune serum or parasites. Free parasites were used in some animals as the infectious inoculum.

Freed parasites were obtained by harvesting the blood of infected adult rats after parasitemia exceeded 50%. "Percentage of parasitemia," as used in this study, is the number of *P. berghei*-infected erythrocytes per 1,000 erythrocytes counted, ×100. These high levels of parasitemia were obtained by pretreatment of the rats with 30 mg of phenylhydrazine · HCl per kg, intraperitoneally, 5 and 3 days before intravenous infection with 0.5 ml of heavily infected blood. On day 3 after infection, the parasitemia exceeded 50%, and the animals were exsanguinated into Alsever solution for the parasite harvest. Parasites were isolated from the blood by a modified continuous-flow ultrasonic treatment procedure (24, 25). The only changes involved the filtration of the sonically treated blood material through a glass-wool pad before centrifugation for 10 min at $1,020 \times g$ to remove unbroken erythrocytes, after which the supernatant was centrifuged for 10 min at $3,300 \times g$ to pellet the parasites. The parasites were then washed twice, suspended in Hanks balanced salt solution (HBSS), and held at 1°C until used, no longer than 2 h. Centrifugations were done in a refrigerated Sorvall RC-2 centrifuge.

In vitro macrophage-parasite adherence tests. CDF rats were anesthetized with ether and killed by exsanguination. Using clean technique, the abdomen was opened and normal, unstimulated macrophages were removed by lavage with 8 to 10 ml of tissue culture medium 199 (M-199; Grand Island Biological Co., Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum and antibiotics. The cells were pelleted by centrifugation for 3 to 5 min at $1,000 \times g$ and resuspended in tris(hydroxymethyl)aminomethane-ammonium chloride buffer [1 part 0.1 M tris(hydroxymethyl)aminomethane (pH 7.2) to 9 parts 0.83% ammonium chloride]. After 10 min in the tris(hydroxymethyl)aminomethane-ammonium chloride buffer at 37°C, the cells were pelleted by centrifugation as above and resuspended in M-199. Cell numbers and viability determinations were made in a standard hemacytometer by counting cells suspended in buffer containing 1% trypan blue stain.

Unstimulated peritoneal washout cells (2×10^6) containing 80 to 85% macrophages, as determined by May-Gruenwald staining characteristics, were suspended in 2 ml of M-199 and incubated in plastic tissue culture dishes (35 by 10 mm) for 30 min at 37°C in an atmosphere of 5% CO_2. Nonadherent cells were then removed by three washes in HBSS, leaving primarily macrophages adherent to the culture dishes.

The macrophage monolayers were sensitized with 1 ml of either normal rat serum (NRS) or HIRS diluted with 2 ml of M-199 and incubated for 30 min at 37°C in an atmosphere containing 5% CO_2. After sensitization, the cells were washed three times in cold HBSS, and 2×10^6 free parasites in 1 ml of cold HBSS were layered over the macrophages and incubated for 30 min. The monolayers were washed three times by immersion in cold HBSS. The monolayers were then fixed with methanol and stained by the Giemsa technique. Two hundred cells showing typical macrophage morphology were counted for the presence and number of adherent parasites on each plate. Each experiment was performed in duplicate, and the data were averaged.

Animals. Male inbred CDF rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used throughout the study. Protection assays were done in weanling rats 4 to 5 weeks of age, and parasites and various sera were obtained from adult rats.

Sera. NRS was harvested from adult CDF rats, aliquoted, and stored at −20°C. HIRS was obtained by challenging rats newly recovered from *P. berghei* infection with 20×10^6 infected CDF rat erythrocytes at weekly intervals for 3 weeks. The animals were bled approximately 2 weeks later, and the sera were separated, pooled, and stored in 2-ml portions at −20°C until needed. Fluorescent serum globulins were prepared from hyperimmune anti-*P. berghei* serum by the method of Cherry et al. (5). Rabbit anti-rat immunoglobulin fluorescein-conjugated immunoglobulin G (IgG) was obtained from Microbiological Associates, Walkersville, Md. Rabbit anti-rat IgG serum for immunoelectrophoresis was obtained from Miles Laboratories, Inc., Elkhart, Ind.

Chromatographic separation of HIRS. Pooled CDF rat anti-*P. berghei* HIRS (4 ml) was dialyzed for 3 days against ethylenediamine acetate buffer and then separated on a QAE-Sephadex A-50 column that

had been swollen in the same buffer. The buffer was prepared as follows: 2.88 g of ethylenediamine acetate was dissolved in 73 ml of glacial acetic acid, and the volume was adjusted to 1 liter with distilled water to give an ionic strength of 0.1 (pH 7.2). Elutions of serum fractions from the column were made with ethylenediamine acetate buffer adjusted to pH 5.0 by adding acetate buffer, and with acetate buffer at pH 4.0. Acetate buffer was made by adding 435 ml of 0.6 M acetic acid to 130 ml of 0.6 M sodium acetate and adjusting the volume to 1 liter with distilled water to give an ionic strength of 0.1 (pH 4.0). Serum fractions were examined and identified by immunoelectrophoresis and by indirect fluorescent antibody technique.

Immunoelectrophoresis. Immunoelectrophoresis was accomplished using 0.65% Difco agar (Difco Laboratories, Detroit, Mich.) in Gelman high-resolution buffer (pH 8.8; Gelman Instrument Co., Ann Arbor, Mich.). The test samples were subjected to electrophoresis at 200 V, 20 mA, for 60 min and then reacted with rabbit anti-rat IgG serum at 25°C in a humidity chamber for 24 h.

Fluorescent antibody techniques. Indirect fluorescent antibody assay for the opsonic antibody was conducted by incubating fixed films of parasitized blood for 30 min at 37°C in HIRS, followed by two washes of 10-min duration in phosphate-buffered saline (pH 7.2). The film was then incubated with rabbit anti-rat immunoglobulin fluorescein-conjugated IgG for 30 min at 37°C. The slides were again washed as above and examined for fluorescence. Indirect fluorescent antibody assay for the macrophage-cytophilic antibody was conducted by incubating macrophage monolayers for 30 min at 4°C in HIRS, followed by two rapid washes in phosphate-buffered saline (pH 7.2). The cells were then incubated with rabbit anti-rat immunoglobulin fluorescein-conjugated IgG for 30 min at 4°C. The cells were washed as before and examined for fluorescence.

Adsorption of HIRS with macrophages. Peritoneal washout cells consisting of at least 80% macrophages were collected from normal CDF rats in M-199, centrifuged at 1,000 × *g* for 2 min, suspended in tris(hydroxymethyl)aminomethane-ammonium chloride buffer for 10 min at 37°C to lyse erythrocytes, centrifuged at 1,000 × *g* for 2 min, and resuspended in M-199. The cells were then plated out in plastic tissue culture dishes (35 by 10 mm) and incubated for 1 h at 37°C in an atmosphere containing 5% CO_2 to promote adherence. Nonadherent cells were washed away after this incubation, and the M-199 was replaced. The plated cells were then held at 4°C until used. After the plate was washed in ice-cold HBSS and the excess was drained, serum was adsorbed by transferring a 1-ml serum sample to the plate, which was then incubated at 4°C for 30 min. At this time, another plate was rinsed in ice-cold HBSS and received the 1 ml of serum from the preceding. The serum was serially adsorbed six times in this manner. Each plate, after use, was washed twice in ice-cold HBSS and then incubated with a 1:10 dilution of fluorescein-conjugated anti-*P. berghei* CDF rat serum globulin for 30 min at 4°C. Each plate was then rinsed three times and examined for specific macrophage fluorescent staining. A negative test indicated blockade of specific macrophage receptors by cytophilic antibody bound during the serial HIRS adsorption. A positive test indicated that no cytophilic antibody remained in the serially adsorbed sample.

Cytophilic antibody elutions. Macrophage-cytophilic antibody was purified by serially adsorbing a 3-ml portion of HIRS six times with 15 × 10^7 peritoneal washout cells in suspension for 30 min at 4°C. The peritoneal washout cells were then incubated at 56°C for 30 min to effect the elution of bound antibody. The supernatant containing the eluted antibodies was adjusted to a 3-ml volume. Both the adsorbed serum and the eluate were tested by indirect fluorescence microscopy for specific activity. The eluate was compared to QAE-Sephadex A-50 fractions 1 and 2 for determination of composition by double diffusion in gel against rabbit anti-rat IgG antiserum.

In vivo protection test no. 1: adsorbed serum. HIRS that had been adsorbed six times by 5 × 10^7 peritoneal washout cells was compared with both NRS and unadsorbed HIRS in 5-week-old male CDF rats. Each test group contained five rats, and each rat received 5 × 10^6 freed parasites in 0.1 ml of HBSS mixed with 0.1 ml of either NRS, HIRS, or adsorbed HIRS and immediately injected intravenously. Percentage-of-parasitemia values were determined by counting 1,000 erythrocytes at random on a Giemsa-stained thin blood film each day. Data were plotted as the daily mean percentage of parasitemia for the group.

In vivo protection test no. 2: eluted antibody. The antibody-containing eluate recovered after 3 ml of HIRS had been adsorbed six times with 15 × 10^7 peritoneal washout cells was compared for protective activity in vivo with the adsorbed serum as well as with both NRS and HIRS in 5-week-old male CDF rats. Each test group contained 5 rats, and each rat received 5 × 10^6 infected CDF erythrocytes suspended in 0.5 ml of HBSS, given intravenously 24 h after intravenous injection of 0.5 ml of either NRS, HIRS, macrophage-adsorbed HIRS, or macrophage eluate. Percentages of parasitemia were determined by counting 1,000 erythrocytes at random on a Giemsa-stained thin blood film each day. Data were plotted as the mean time required for the group to reach 1% parasitemia.

In vivo protection test no. 3: HIRS fractions. Fractions of HIRS that had been separated by elution chromatography on QAE-Sephadex A-50 were compared with both NRS and HIRS in 5-week-old male CDF rats. Each group contained 5 rats, and each rat received 0.1 ml of NRS, HIRS, fraction 1, 2, or 3, or 0.1 ml of fraction 1 plus 0.1 ml of fraction 2. The infectious inocula consisted of 5 × 10^6 freed parasites suspended in 0.1 ml of HBSS, which was mixed with the serum or serum fraction(s) and immediately injected intravenously via the lateral tail vein. Percentages of parasitemia were determined by counting 1,000 erythrocytes at random each day on a Giemsa-stained thin blood film. Data were plotted as the mean time required for the group to reach 1% parasitemia.

RESULTS

In vitro macrophage-parasite adherence tests. The effects of arming the macrophages with macrophage-cytophilic antibody specific

for *P. berghei* parasites before their incubation with free parasites are presented in Fig. 1. Of the macrophages pretreated with HIRS, 25% were associated with adherent parasites, as opposed to only 9% of those pretreated with NRS. Of the actual parasites counted, more than three times as many parasites were found to be associated with macrophages pretreated with HIRS as with macrophages pretreated with NRS. Those macrophages pretreated with NRS that showed positive adherence results usually had one to three adherent parasites, whereas macrophages pretreated with HIRS that showed positive adherence results usually had two to six adherent parasites. Analysis by Student's *t* test showed a significant difference between NRS and HIRS for both the percentage of macrophages positive and the number of adherent parasites per 200 macrophages ($P < 0.025$).

Chromatographic separation of HIRS. Figure 2 is an optical density tracing at 280 nm recorded during the elution of fractions of HIRS from a QAE-Sephadex A-50 column at pH values of 7.2, 5.0, and 4.0 (fractions 1, 2, and 3, respectively). When examined by immunoelectrophoresis, fractions 1 and 2 were found to consist of IgG_1 and IgG_2, respectively, following the nomenclature of Bazin et al. (2), whereas fraction 3 was found to contain albumin and IgM.

Adsorption of HIRS with macrophages. HIRS (1 ml) adsorbed four times by 5×10^6 peritoneal washout cells was found to block subsequent direct fluorescent antibody staining of macrophages; after the fifth adsorption it partially blocked the fluorescent antibody staining of the macrophages, and after the sixth adsorption it did not block the direct fluorescent staining of the macrophages. Thus, the macrophage-cytophilic antibody was no longer present at inhibitory levels in 1 ml of HIRS after six adsorptions with 5×10^6 peritoneal washout cells.

Cytophilic antibody elutions. When the eluate from peritoneal washout cells that had been used to adsorb HIRS was compared by the indirect fluorescent antibody technique to the adsorbed HIRS, it was found that the eluate contained primarily macrophage-cytophilic antibodies, whereas the adsorbed HIRS contained primarily opsonic antibodies. When the same eluate was compared by double diffusion in gel to fractions 1 and 2 obtained by QAE-Sephadex A-50 chromatography of HIRS, fraction 1 was shown to be identical with the antibodies eluted from the macrophages, whereas fraction 2 was shown to contain antibodies distinct from both the eluate and fraction 1, although all were identified as belonging to the IgG class (Fig. 3). Thus, fraction 1 has been shown to provide

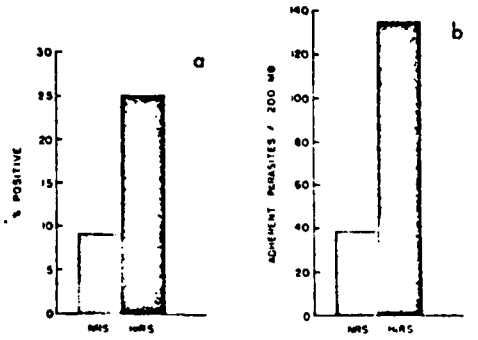

FIG. 1. *Effects of cytophilic antibodies upon the ability of macrophages (Mφ) to retain free P. berghei parasites. (A) Parasite-macrophage adherence (percentage of macrophages showing adherence). (B) Number of parasites adherent per 200 macrophages.*

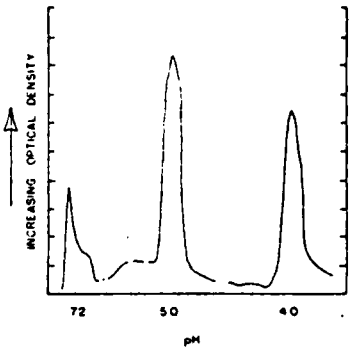

FIG. 2. *pH elution pattern of HIRS from QAE-Sephadex A-50.*

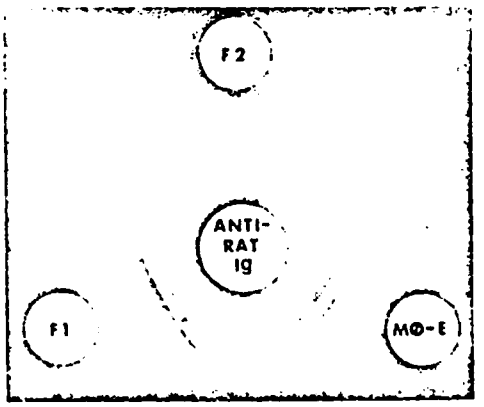

FIG. 3. *Precipitin pattern formed in gel by eluted macrophage-cytophilic antibodies and QAE-Sephadex HIRS fractions when reacted against anti-IgG antiserum.*

separation of those antibodies that are capable of macrophage-cytophilic activity from the opsonizing antibodies that are contained in fraction 2. Indirect immunofluorescent tests have verified that macrophage-cytophilic antibodies are contained only in fraction 1, and that parasite opsonizing antibodies are found in fraction 2, but not in fraction 1.

In vivo protection test no. 1: adsorbed serum. The protective effects of HIRS that had been depleted of macrophage-cytophilic antibodies was shown to be reduced from those exhibited by unadsorbed HIRS (Fig. 4), as demonstrated by the earlier onset and rise of parasitemia after the challenge infection was initiated. Analysis by Student's t test showed a significant difference between the adsorbed and the unadsorbed HIRS groups on day 10 ($P < 0.05$).

In vivo protection test no. 2: eluted antibody. When eluted macrophage-cytophilic antibodies were compared with HIRS depleted of macrophage-cytophilic antibodies, it was found that the protective capacity of HIRS depleted of macrophage-cytophilic antibodies was diminished, and that the eluate which contained only the eluted macrophage-cytophilic antibodies had no protective capacity (Fig. 5). Data obtained subsequent to these experiments indicated that recovery of macrophage-cytophilic antibody was at an efficiency of approximately 58%; therefore, a quantitative reduction of cytophilic antibody occurred in the eluate as compared with unadsorbed serum. This would be expected to dimish any observed effects of cytophilic antibody when compared to the original volume. However, the magnitude of the reduction in activity of the adsorbed serum is such that it would be reasonable to expect some effect from 58% of the original amount if it were capable of independent protective activity. No significant difference was seen between the eluate and NRS, but Student's t test showed a significant difference between the macrophage-adsorbed HIRS and unadsorbed HIRS ($P < 0.025$).

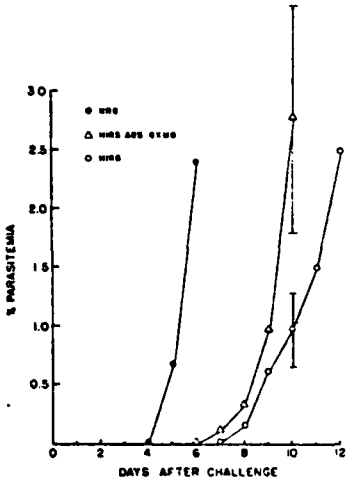

FIG. 4. *Effect of adsorption with macrophages upon the protective capacity of P. berghei immune serum in rats. Values plotted are means (± standard error of the mean) obtained from counts of 1,000 cells on each film (n = 5).*

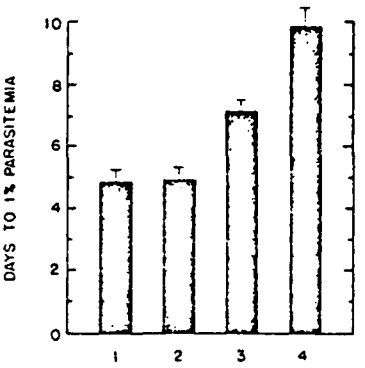

FIG. 5. *Protective effects against P. berghei of macrophage-adsorbed and macrophage-eluted HIRS fractions in rats. Values plotted are means (± standard error of the mean) obtained from counts of 1,000 cells on each film (n = 5). Rats received 0.5 ml of (1) NRS, (2) macrophage eluate, (3) macrophage-adsorbed HIRS, or (4) HIRS intravenously 24 h before infection.*

In vivo protection test no. 3: HIRS fractions. Fraction 1, containing the macrophage-cytophilic antibodies, had no protective capacity against *P. berghei* infection in vivo, and fraction 2 contained only moderate protective capacity. However, when fractions 1 and 2 were recombined in vivo, a synergistic effect was seen, resulting in a greater protective capacity than would be indicated by the expected additive effects of the individual fractions. Fraction 3 contained minimal protective capacity, which can be ascribed to the presence of IgM antibodies (Fig. 6). Analysis by Student's t test showed a significant difference between the time required for the group that received fraction 2 and the group that received fraction 1 and 2 combined to reach 1% parasitemia ($P < 0.05$).

DISCUSSION

Our findings indicate that there are two major and distinct types of IgG antibody that provide protection against *P. berghei* in rats. The first of these is an opsonic antibody, which has as its specific ligand some site on the parasite sur-

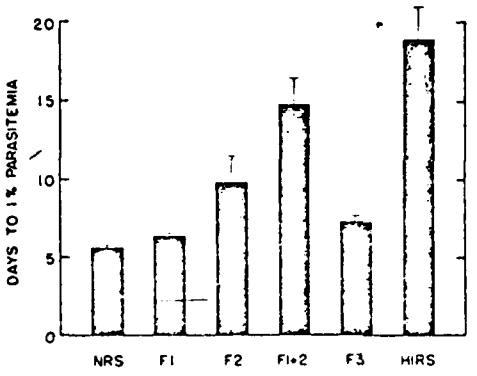

FIG. 6. *Protective effects against P. berghei in rats of QAE-Sephadex HIRS fractions. Values plotted are means (± standard error of the mean) obtained from counts of 1,000 cells on each film (n = 5). F1, Fraction 1 (cytophilic); F2, fraction 2 (coating); F1+2, 0.1 ml of each; F3, fraction 3 (albumin with or without coating).*

face. The second is a macrophage-cytophilic antibody that is unable to bind to parasites until after it has become attached to the surface of a macrophage. Sodoman and Haferkamp (27) reported finding in rats such macrophage-cytophilic antibodies to tuberculoprotein after BCG immunization and after appropriate immunization to group A *Streptococcus* carbohydrate also. Binding of the cytophilic antibody to the macrophage surface is probably by an Fc receptor (8, 10). Davey and Asherson (8) concluded that the macrophage receptor for macrophage-cytophilic antibodies against sheep erythrocytes in guinea pigs might be a phospholipid or phospholipoprotein, and Arend and Mannik (1) found that cytophilic antibody receptors on macrophages were destroyed by phospholipase. We have not yet attempted to determine the nature of the receptors in our system.

Upon attachment to the macrophage surface membrane, the antibodies presumably undergo steric rearrangement, whereupon they become capable of specifically binding parasite receptors. However, the protective nature of this interaction is not manifested in the absence of specific opsonic antibody. Indeed, it is well established that some cytophilic antibodies fix antigens to macrophages without inevitably stimulating phagocytosis (31). Levenson and Braude (17) observed that anti-*S. typhi* Vi polysaccharide obtained early in immunization could induce rosette formation of Vi-coated erythrocytes around macrophages, and that prolonged incubation of these rosettes at 37°C did not lead to phagocytosis. Internalization may be dependent upon the presence of complement-fixing antiparasite antibodies at the macrophage-parasite interface. Ehlenberger and Nussenzweig (11) have recently demonstrated synergy between Fc and C3 receptors in the phagocytic process. Bianco et al. (3) have concluded that the process of phagocytosis is accompanied by a change in the macrophage C3b receptor that allows a shift in function from binding to both binding and internalization.

Miller et al. (21) have recently reported a lack of correlation in monkeys between functional immunity in vivo and the presence of antibodies capable of inhibiting merozoite invasion in vitro. In the immune response of rats to *P. berghei*, a synergistic relationship exists between the macrophage-cytophilic and the opsonic antibodies wherein the combined protective effect is greater than the sum of the individual protective capacities. The exact nature of this interaction remains unclear. It has been proposed (7, 20) that the antibody coating the parasites may interfere with penetration of the erythrocyte by the merozoite form of the parasite. We have observed in this study that the opsonic antibody is necessary for the manifestation of the protective effect of the macrophage-cytophilic antibodies. Perhaps the macrophage-cytophilic antibodies promote only the adherence of parasites to the phagocytic cells, and not their subsequent ingestion. Stossel (29) has observed that, under differing conditions, "some particles stick tightly to phagocytes but are not ingested, some stick and are partly ingested, and still others stick and are completely ingested." The opsonic antibodies may trigger the actual ingestion of the parasites by the macrophages, but may be less efficient alone in promoting adherence. It has been stated that internalization of particles attached to macrophages is triggered by IgG, and that different roles are assigned for the macrophage receptor sites for complement (C3) and for immunoglobulin (19, 26).

Destruction of the infective merozoites is necessary to control an infection by malarial parasites. Preliminary electron microscopic studies under way in this laboratory by C. Brooks have indicated that, although trophozoites in a free-parasite preparation will adhere to macrophages in the presence of either normal or immune serum, merozoites are found to be adherent to macrophages only in the presence of immune serum. A capsule has been demonstrated as present on the infective merozoite stage of *Plasmodium* (20). This capsule may very well confer antiphagocytic properties upon the merozoite. Immune serum has been shown to contain protective antibodies which bind to this capsule (20), yet these antibodies are not capable of direct merozoite neutralization. However, these

antibodies may serve to reduce or eliminate the antiphagocytic nature of the capsular material by reduction of the hydrophilic characteristics of the capsular material, thereby favoring the physical processes of phagocytic engulfment (22); by stabilization of the amorphous capsular material, thereby facilitating adhesion and subsequent engulfment; or by both mechanisms.

Although the macrophage-cytophilic antibodies may facilitate the attachment of the merozoites to the macrophage, alteration of the capsular material (perhaps by complement fixation) may not occur in the absence of the opsonic antibody, and ingestion of the parasite would therefore be less likely. Furthermore, should the merozoite be swept loose, leaving some capsular material attached at the macrophage surface, the recognition capability of the cytophilic antibody-armed macrophage would be reduced. Thus it becomes apparent that cytophilic antibody-mediated attachment of infective merozoites would result in enhancement of the protective effects of the opsonic antibody, but would probably not be protective alone.

Previous work in this laboratory by Hamburger and Kreier (14) has demonstrated that passive immunity to malaria cannot be conferred to mice by rat-origin immune serum, with the implication that the site of protective action of antimalarial antibodies is species specific. This action is probably at the level of phagocytosis, i.e., the macrophage. Further work (13) suggested that the parasite opsonizing antibodies were of low avidity and quickly became dissociated from the parasites in vivo, or that a cytophilic antibody was involved in phagocytosis, or both. Low opsonizing-antibody avidity was demonstrated by these authors in a subsequent publication (15), and now we have additionally demonstrated the presence and role of a macrophage-cytophilic antibody.

ACKNOWLEDGMENTS

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Role of the Surface Coat in In Vitro Attachment and Phagocytosis of *Plasmodium berghei* by Peritoneal Macrophages†

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Evidence is presented to indicate that *Plasmodium berghei* merozoites, but not trophozoites, have an antiphagocytic capsule. The capsule appears to form around the developing merozoites of the schizont in the parasitophorous vacuole. Serum from animals immune to *P. berghei* reacts with this capsule. After reaction with immune serum, the antiphagocytic action of the capsule is lost. By the process of binding serum protein, the capsule becomes electron dense and can be readily visualized as the surface coat by electron microscopy. At physiological temperatures, phagocytosis by macrophages rapidly follows adhesion of antibody-coated parasites. Both tight and loose phagosomes are formed.

The ability of a host to overcome malaria infection depends partially on the phagocytic system. The frequency and effectiveness of parasite-erythrocyte contact as compared to the frequency and effectiveness of parasite-macrophage contact probably determines the outcome of the infection (11). The experimental demonstration of the phagocytic process has been difficult. This has been because free parasites were not available for study and when they were available, the experimental results obtained in phagocytic studies have been inconsistent. There has been, for example, much phagocytosis with normal serum in some experiments and only a quantitative additional effect with immune serum (3).

In the last few years some of the technical problems inhibiting the study of the interaction of parasites, antiserum, and phagocytes have been overcome. Specialized adaptations of culture techniques have permitted the harvest of mature merozoites of *Plasmodium knowlesi* (19), and improvements in culture (25) may soon permit the harvest of merozoites of *P. falciparium* as well, while a continuous-flow sonic oscillation technique (15, 16) yields free parasites in any of the forms parasitizing erythrocytes (13).

In this study we used a mixed population of trophozoites and merozoites freed from rat erythrocytes by sonification to study the role of immunoglobulin and stage of development of the parasites in attachment and phagocytosis of parasites by peritoneal macrophages in vitro.

† Army Malaria Project paper no. 1495.
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(This work is part of a thesis submitted by Carolyn Brooks as partial fulfillment of the requirements for a doctorate at the Ohio State University).

MATERIALS AND METHODS

Animals. Rats utilized for serum and parasite and macrophage harvests were inbred Fisher 344 (CD*F) males obtained from Charles River Breeding Laboratories, Wilmington, Mass. They were housed in the animal facility of the Department of Microbiology, The Ohio State University. Food and water were given ad libitum.

Parasites. The strain of *P. berghei* used originated from the Walter Reed Army Institute of Research and was maintained in our laboratory by serial passage in rats and mice or by storage in Alsever solution plus 10% (vol/vol) glycerol in liquid nitrogen (−193°C).

Harvesting of free parasites. Mature rats were used for harvesting parasites. To insure high parasitemias in these animals, it was necessary to increase the reticulocyte count by pretreatment with phenylhydrazine (11). When the parasitemia reached at least 50% and contained a high proportion of schizonts, the blood was harvested into cold Alsever solution by cardiac puncture. The blood was centrifuged at 650 × *g* for 10 min at 5°C. The supernatant fluid was removed by aspiration, and the cells were resuspended and washed twice more in cold Alsever solution. Thereafter, leukocytes were removed by filtration of a 10% suspension of washed erythrocytes through columns of packed powdered Whatman no. 1 filter paper over a plug of glass wool. The filtered erythrocytes were disrupted by treatment in a continuous-flow sonification system by methods previously described (15, 16). The sonificated material was centrifuged at 650 × *g* to remove the unlysed erythrocytes. The supernatant was then centrifuged at 1,450 × *g* for 10 min and the parasites collected were washed by an

additional centrifugation in Alsever solution.

Hyperimmune serum. Rats recovered from *P. berghei* received monthly reinoculations of infected erythrocytes from other inbred rats of the same strain. Sera collected 2 weeks after the last injection were separated, pooled, and stored at −20°C.

Macrophage collection. Rats were anesthetized with ether and then bled by cardiac puncture for normal serum. To obtain macrophages, the skin over the viscera was removed, the peritoneum was snipped, and the cavity was filled with sterile medium 199 (Microbiological Associates). The filled abdominal cavity was massaged, and the fluid was withdrawn into sterile pipettes and placed into sterile siliconized culture tubes. The macrophages were collected by centrifugation.

Parasite surface coat formation. For study of surface coat formation by electron microscopy, ultrasonically freed parasites were prepared by incubation at 37°C or in an ice bath in 5% hyperimmune or 5% normal serum or Alsever solution for 5 or 15 min.

Attachment studies. Attachment studies were carried out by light or electron microscopy. For both light and electron microscopic studies, parasites were exposed to rat origin peritoneal macrophages in suspensions in test tubes. For light microscopy, thin film preparations were made and stained by the Giemsa method. The techniques for staining were similar to those previously described (12).

Preliminary attachment studies revealed that 10 min of incubation at 4°C permitted attachment of parasites to macrophages in our system. After 10 min of incubation, the suspensions were removed and the pelleted cells were fixed and prepared for either light or electron microscopy.

The various combinations of macrophages, parasites, and normal or immune sera which were used in attachment and phagocytosis studies are listed in Tables 2 and 3. Parasitized erythrocytes were also studied in the same manner.

Phagocytosis. After 10 min of incubation in the cold to allow attachment, portions of macrophages and parasites prepared as previously described were placed in a 37°C water bath for 5- to 15-min intervals. At the appropriate times, the tubes were spun in a refrigerated centrifuge and the pellets were fixed for electron microscopy.

Electron microscopy. Parasite and macrophage preparations were fixed in 1.25% glutaraldehyde, 0.05 M phosphate buffer, pH 7.3, and 0.116 M sucrose. After washing in 0.05 M phosphate buffer, the preparations were postfixed in 1% osmium tetroxide for 1 h, washed, dehydrated in ethanol and propylene oxide, and embedded in an Epon 812 mixture. Sections were cut on a Sorvall Porter-Blum ultramicrotome equipped with a glass knife. The sections were mounted on uncoated 300-mesh copper grids, and stained with uranyl and lead citrate. The sections were examined on a 9S-2 Zeiss microscope.

RESULTS

Surface coat formation. Most of the merozoites present in sonically freed parasite preparations developed a thick electron-dense surface coat upon incubation for 15 min in immune serum (Fig. 1). Trophozoites present in the same preparations were often agglutinated by the immune serum, but did not develop a similar thick electron-dense surface coat (Fig. 2). Incubation in normal serum caused formation of a thin surface coat on most merozoites, but had no visible effect on trophozoites. Schizonts freed from erythrocytes also developed a thick surface coat upon incubation in immune serum (Fig. 3). Many of the merozoites incubated in immune serum for 5 min had an electron-lucid area (Fig. 4) between the electron-dense surface coat and the plasmalemma, whereas the surface coat surrounding the merozoites incubated in immune serum for 15 min was in direct contact with the plasmalemma.

Effect of addition of immune serum to macrophage-parasite mixtures. In macrophage-parasite preparations held at 5°C, more macrophages bound parasites in the presence of immune serum than in the presence of normal serum or in the absence of serum. The average number of parasites on the macrophages was also greater in the presence of immune serum than in the presence of normal serum or in the absence of serum. These results were similar

FIG. 1. *Thin-sectioned merozoite (M) that has been incubated in medium containing 5% hyperimmune serum for 15 min at 37°C. The parasite developed a very thick surface coat (SC).*

FIG. 2. *Trophozoites (T), which were incubated in 5% hyperimmune serum, with small amounts of material on their surfaces but no surface coat similar to the merozoites. The trophozoites were agglutinated by the immune serum.*

whether the parasites and serum were added to macrophage suspensions which were subsequently fixed, Giemsa stained, and examined by light microscopy or were prepared for examination by thin-section electron microscopy (Table 1).

Relative roles of cytophilic and opsonic components in immune serum on parasite attachment to macrophages. In macrophage preparations held at 5°C, more macrophages bound parasites which had been pretreated with immune serum than those pretreated with normal serum or not pretreated. The average number of antibody-pretreated parasites bound per macrophage was also greater than was the average number of normal serum-pretreated or untreated parasites. Pretreatment of the macrophages with immune serum enhanced their capacity to bind untreated parasites, but to a lesser degree than did pretreatment of parasites. Pretreatment of both macrophages and parasites with immune serum enhanced the degree of attachment of parasites to macrophages more than did treatment of parasites or macrophages alone (Table 2).

Effect of immune serum treatment upon the development stage of free parasites bound to macrophages. It was not possible to differentiate between merozoites and small free trophozoites by light microscopic examination of Giemsa-stained preparations of sonically freed parasites. This differentiation was, however, made by thin-section electron microscopy. When the parasites attached to macrophages were classified according to whether they were trophozoites or merozoites, it became apparent that practically all of the parasites which attached to macrophages in the presence of normal serum or the absence of serum were trophozoites (96–100%), whereas in the presence of immune serum, substantial numbers of merozoites (12 to 29%) also adhered to macrophages (Table 2).

Relative roles of cytophilic and opsonic components in immune serum on parasite ingestion by macrophages. Cytophilic antibody which facilitates attachment of parasites to macrophages may not facilitate their internalization. In fact, unopsonized parasites attached to macrophages by cytophilic antibody may come off upon prolonged incubation at 37°C (Table 3, lines 1 and 2). Attached parasites which have been opsonized, i.e., coated with antibody, on the other hand, are internalized within 5 min when the temperature of the cultures is raised to 37°C from 5°C (Table 3, lines 3, 4, 5, 6).

Process of phagocytosis of attached parasites. Parasites are taken into the cytoplasm of macrophages which initially project thin pseudopods around the parasite (Fig. 5). The pseudopods begin ingestion with such avidity that parasites may be distorted in the process. The cytoplasm around the forming phagosome is usually devoid of organelles. Parasite-containing vacuoles are found at random locations in the cytoplasm of macrophages; some lie in the area of the nucleus and others are situated peripherally, near the cell membrane. The phagosomes could be seen in two forms, the tight phagosome (Fig. 6) and the loose phagosome (Fig. 7). The tight phagosomes are those in which the phagosomal membrane is directly opposed to the cell membrane of the engulfed organism. The loose type has a wide space between the membrane of the phagosome and perimeter of the organism. Generally, plasmodia observed in loose phagosomes are relatively unaltered. In some loose phagosomes the space was occupied by flocculent material of moderate density; in others the space appeared completely electron lucent. Some of the vacuoles containing parasites were lined by electron-dense material.

Phagocytosis of erythrocytes. In vitro

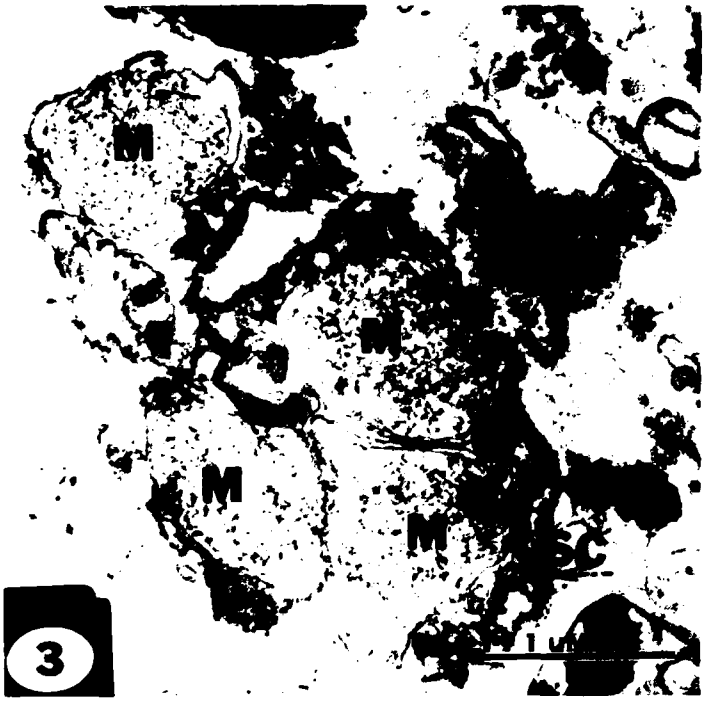

FIG. 3. *The matrix of the parasitophorus vacuole of this free schizont still partially surrounds the developing merozoites (M). The portion of the matrix which adheres to the parasites has reacted with the immune serum in which the schizont was incubated and formed a thick surface coat (SC). A clump which may be surface coat material appears to be partially shed from the schizont (CSC).*

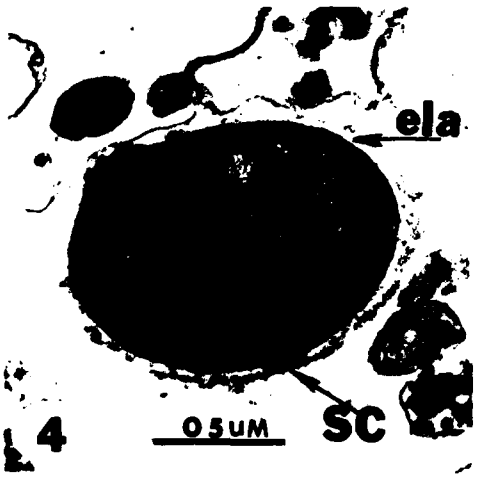

FIG. 4. *An electron-lucid area (ela) is present between the dense surface coat (SC) and the plasma membrane on this merozoite, which was incubated for 5 min in 5% hyperimmune serum at 37°C.*

phagocytosis of erythrocytes, either infected or noninfected, was a very rare event. The rareness of erythrophagocytosis was not affected by any serum treatment used.

DISCUSSION

The presence of a surface coat on merozoites of plasmodia was first reported by Ladda et al. (14). The existence of this surface coat on merozoites which have been exposed to immune serum and even to normal serum has been amply confirmed (1, 18). It has usually been assumed that this coat would be absent from trophozoites, but its absence has not been demonstrated because previous studies were done on parasite preparations which did not contain free trophozoites. The function of this surface coat has not been determined, nor has its origin.

Mason et al. (17) have suggested that the surface coat is of parasitic origin and that it is secreted after the merozoites are released. They showed a schizont inside a parasitophorous vacuole which they considered to have no surface

TABLE 1. *Effect of added immune (IS) or normal serum (NS) upon attachment of free P. berghei parasites to peritoneal macrophages in cultures incubated at 5°C*

Serum	Type of microscopy	No. of macrophages examined	No. of parasites on macrophages		No. of macrophages with parasites attached	
			Total	Per macrophage	Total	%
IS	Light	822	1,260	1.53	658[a]	80
	Electron	110	204	1.85	92[a]	84
NS	Light	723	745	1.03[a]	412[a]	58
	Electron	84	109	1.30	56[a]	67
None	Light	562	382	0.68	242	43
	Electron	34	60	0.71	43	51

[a] Average number of macrophages binding parasites and number of parasites bound per macrophage is significantly greater ($P < 1\%$) in these immune serum treatment groups than in the corresponding normal serum groups (Z test for equality of two proportions; 9).

TABLE 2. *Effect of various immune serum (IS) or normal serum (NS) treatments of parasites and macrophages on the percentage of macrophages showing attached free parasites, average number of attached free parasites per macrophage, and development stage of free parasites which attach to macrophages, as determined by thin-section electron microscopy*

Treatment	No. of macrophages examined	% Macrophages with attached parasites	Attached parasites		Attached merozoites		Developmental stage of attached parasites (%)	
			Total	Per macrophage	Total	Per macrophage	Trophozoites	Merozoites
IS added to parasite-macrophage culture	110	84	212	1.93	62[a]	0.56	71	29
NS added to parasite-macrophage culture	84	66	106	1.27	0	0	100	0
Macrophages pretreated with IS	90	72	147	1.63	18[a]	0.20	88	12
Macrophages pretreated with NS	83	58	108	1.30	0	0	100	0
Parasites pretreated with IS	90	93	216	2.40	41[a]	0.46	81	19
Parasites pretreated with NS	90	57	81	0.90	2	0.03	98	2
Parasites and macrophages pretreated with IS	100	96	242	2.42	51[a]	0.51	79	21
Parasites and macrophages pretreated with NS	72	60	67	0.93	3	0.04	96	4
All IS groups	390	86	817	2.09	172[a]	0.46	80	21
All NS groups	329	60	362	1.10	5	0.02	99	1
No serum in culture	87	47	69	0.79	0	0	100	0

[a] Number of merozoites attached to macrophages in the presence of immune serum is significantly greater ($P < 1\%$) than the number of merozoites attached to macrophages in the presence of normal serum in all treatment groups (Z test for equality of two proportions; 9).

coat, although it obviously was in a relatively low density matrix which appeared to serve as a spacer between the component parts of the schizont and the membrane of the parasitophorous vacuole. Figure 3 shows a schizont similar to the one shown by Mason et al. (17). This schizont, however, has been ripped from its vacuole by sonification and exposed to immune serum. It has a thick coat which occupies a part of the area filled by the matrix in the parasitophorous vacuole. A mass (CSC) which has the appearance of surface coat material can be seen partially detached from the schizont (Fig. 3). The nature of this material cannot be proven by techniques currently available, and, thus, alternative interpretations of its nature are possible.

The presence of an electron-lucid area between the surface coat and the plasmalemma in

TABLE 3. *Effects of various immune (IS) and normal (NS) serum treatments upon the percentages of macrophages showing phagocytosis of free parasites after 5 or 15 min of incubation at 37°C after 10 min of incubation at 5°C to permit parasite attachment*

Treatment	No. of macrophages examined	% Macrophages with no attached or phagocytozed parasites		% Macrophages with attached parasites only		% Macrophages with ingested parasites	
		5 min	15 min	5 min	15 min	5 min	15 min
Macrophages pretreated with IS	102	26	50	44	18	29	31
Macrophages pretreated with NS	92	53	60	17	8	31	32
Parasites pretreated with IS	109	7	10	5	0	88	90
Parasites pretreated with NS	88	31	30	32	21	38	49
Parasites and macrophages pretreated with IS	84	4	3	7	19	89	91
Parasites and macrophages pretreated with NS	91	33	37	6	12	48	51
No serum in culture	296	55	62	21	13	25	29

FIG. 5. *Both the parasite and macrophage were sensitized in 5% hyperimmune serum. The pseudopodia (PS) of the macrophage have begun to ingest the merozoite (M). The merozoite has a distinct surface coat (SC) which is also being ingested.*

parasites incubated for a short time in immune serum (Fig. 4) but its absence from parasites subjected to longer incubation (Fig. 1) is best explained by postulating a slow penetration of antibody into a loose gel surrounding the parasites rather than by postulating a secretion of capsule during incubation.

It is well known to bacteriologists that rough

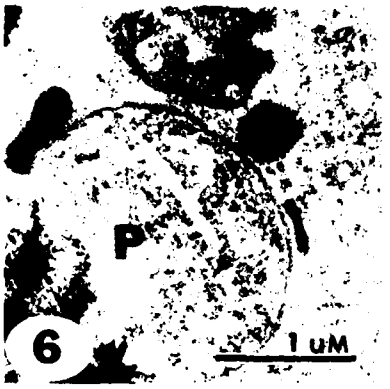

FIG. 6. *Tight phagosome in which the phagosomal membrane is directly apposed to the cell membrane of the engulfed organism (P)*

(i.e., nonencapsulated) strains of pathogenic bacteria are avirulent and easily phagocytized, whereas smooth (i.e, encapsulated) strains are virulent. The capsule of virulent pneumococci swells on exposure to immune serum (the Quellung reaction) (5). The pneumococcal capsule is electron lucid, but becomes electron dense when it imbibes serum protein, particularly specific immunoglobulin (21). The surface coat of the plasmodial merozoite which becomes visible after incubation of the parasite in serum could be an electron-lucid merozoite capsule which has imbibed serum protein in a fashion analogous to the imbibition of serum protein by the pneumococcal capsule.

In the present studies we observed that merozoites (like virulent smooth bacteria) were rarely bound to or ingested by macrophages in the absence of immune serum, whereas trophozoites (like rough avirulent bacteria) were both bound to and ingested by macrophages in the presence of normal serum or even in the absence of serum. Trophozoites do not have a surface coat after exposure to serum and thus probably do not have a capsule. The trophozoite is normally intracellular and thus does not need an antiphagocytic defense, whereas the merozoites must pass from cell to cell and would find an antiphagocytic capsule of considerable utility. This work thus indicates that the function of the merozoite capsule is that of an antiphagocytic defense. If this capsule is impregnated with normal serum, it could provide a further disguise for the parasite. On the other hand, when the capsule is impregnated with specific antibody, its antiphagocytic defense is overcome.

Ladda et al. (14), Miller et al. (18), and Bannister et al. (1) have shown that the surface coat is pushed back and released during penetration of erythrocytes by merozoites. When high parasitemias are present and large numbers of merozoites are released, released surface coat material from penetrating merozoites could react with cytophilic antibody on the macrophage membranes and with opsonic antibody and block macrophage function.

Cochrane et al. (4) found that surface coat material on plasmodial sporozoites migrated to the posterior pole and called this phenomenon capping. Doyle et al. (7) suggested that a similar process occurred with plasmodial merozoites. Our data (Table 3) suggests the possibility that merozoites whose capsules are not stabilized by antibody may disassociate from their surface coats and so escape phagocytosis. Use of the term capping by Doyle et al. (7) and Cochrane et al. (4) to describe the movement of the surface coat material of protozoa is generalizing the term extensively. DePetris and Raff (6) coined the

FIG. 7. *Loose phagosome in which a wide space exists between the membrane of the phagosome and perimeter of the ingested organism (P). The space is occupied by fluocculent material, but is mostly electron lucid.*

term "capping" to describe a process of agglutination of elements in a fluid plasmalemma. The process observed with plasmodial merozoites would better be called agglutination of shedding of capsular material.

The infrequency with which macrophages in in vitro preparations ingest erythrocytes from animals with malaria was noted before (3, 12). As erythrocyte ingestion occurs in vivo during acute malaria, some factor other than simple recognition of surface alteration must play a role in this ingestion. A reduction in cell elasticity (24), a necessary requirement for passage of cells through reticuloendothelial organs, may be in part responsible for erythrophagocytosis in vivo.

Our attachment studies were performed in the cold to disassociate attachment from ingestion. We studied ingestion by raising the temperature of the cultures. It was because of this technique that we were able to observe that cytophilic antibody does not facilitate ingestion, only adhesion. A discussion of the characteristics and roles of opsonic and cytophilic antibody in immunity to rodent plasmodia can be found in the paper by Green and Kreier (10). In that paper the immunoglobulin classes of the antibodies involved in stimulation of phagocytosis of plasmodia and the effects of absorption by parasites and macrophages on the phagocytosis-stimulating capacity of the immune serum are described. After attachment, ingestion of antibody-coated parasites occurred very rapidly when the culture temperature was raised. Some of the parasites appeared in tight, and some in loose, phagosomes. Parasites in tight phagosomes appeared relatively unaltered.

Whether loose phagosomes represent deficient phagocytosis is unclear; Dumont and Robert (8) found that when the fungus *Histoplasma* was enclosed in loose phagosomes, it was morphologically unaltered and oftentimes had undergone budding, which would indicate that this type of phagosome rendered the host cell defenseless against the organism. Belcher et al. (2), studying *Candida albicans*, found disrupted organisms within both tight and loose phagosomes. We did not observe any correlation between phagosome type and presence of capsular material or antibody. The roles of the loose and tight phagosomes in phagocytic clearance of plasmodia need more study.

An electron-dense zone was seen to surround some ingested plasmodia. The electron-dense zone surrounding some of the ingested parasites is not peculiar to plasmodia. Belcher et al. (2) suggest that in *Candida* it is of parasite origin. They suggested that it was parasite material which separated from the *Candida* and accumulated on inner surface of the phagosome. Dumont and Robert (8) considered the electron-dense zone to be of macrophage origin and postulated that it was composed of lysosomal contents.

In an earlier study we showed that the washings from free parasites provided an immunizing agent against challenge with blood origin parasites (23). In these studies we have demonstrated an antiphagocytic function for the merozoite capsule, the presence of capsular material on schizonts, and a role for humoral antibody in overcoming the capsular antiphagocytic function. These observations thus explain why capsular material is an antigen for immunization and why merozoites (20) and schizonts (22) are important in vaccines against plasmodia.

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THE ISOLATION OF A SOLUBLE COMPONENT OF *PLASMODIUM BERGHEI* WHICH INDUCES IMMUNITY IN RATS

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SUMMARY

Soluble material was obtained from sonically freed plasmodia by three procedures. Two procedures, cryo-impacting and freeze-thawing were evaluated for their ability to disrupt the parasites and release soluble material. The soluble material obtained by these procedures was compared to material washed from the surfaces of sonically freed parasites. Between 35 and 40% of the total parasite protein was solubilized by freeze-thawing or cryo-impacting. One cycle of freeze-thawing released nearly as much protein as could be released by this method and additional cycles of freeze-thawing had little additional effect. Cryo-impacting solubilized only a small amount of protein in addition to that which was released by the cycle of freeze-thawing inherent in the procedure. Reductions in the packed cell volume of the material remaining after freeze-thawing or cryo-impacting indicate that the insoluble fragments are broken into smaller pieces as treatment is extended.

Electron microscopy of 30 second cryo-impacted and three times freeze-thawed parasites revealed membrane fragments similar in appearance. Patterns obtained by polyacrylamide gel electrophoresis of the soluble material from freeze-thawed and cryo-impacted parasites were also similar and approximately 13 protein bands were demonstrated. The material washed from the surfaces of the free parasites, on the other hand, resolved into only two to four major bands on the gel columns.

In immunization studies, the soluble and insoluble fractions obtained by freeze-thawing or cryo-impacting and the material washed from the surfaces of the parasites all stimulated a protective immune response. On the basis of the amount of protein required to stimulate roughly comparable immunity, the soluble fraction obtained by freeze-thawing or cryo-impacting free parasites

was about twice as potent an immunogen as was the insoluble fraction. The material obtained by gentle washing of the freed parasites was approximately 20 times as potent an immunogen as were the freed parasites and about 7 times as potent as the soluble material obtained by freeze-thawing or cryo-impacting.

INTRODUCTION

Fractionation of malaria parasites into their component parts is desirable for many serological, biochemical and immunological studies. Most techniques currently used for fractionation require at least some degree of solubilization before separation can be effected. A major objective of research on plasmodia is to obtain a purified antigen which will stimulate the host to develope a protective immunity. To do this, solubilization, fractionation and characterization of the isolated materials are necessary.

Freeze-thawing is one of the most widely used methods for obtaining soluble materials from cells. Free plasmodia and infected red cells have been treated with multiple cycles of freeze-thawing to release soluble components (Sherman and Hull, 1960; Kreier et al., 1976). Cryo-impacting has been shown to be effective in the disruption of bacterial cells and even in disruption of the endospores of Bacillus megaterium (Smucker and Pfister, 1975). Cryo-impacting subjects the material to a physical pounding while it is frozen in liquid nitrogen. Techniques that have been used to obtain soluble extracts of plasmodia have been reviewed by Kreier (1977).

In an earlier report we described an antigen with protection stimulating characteristics which had been obtained by gentle washing of sonically freed parasites (Saul and Kreier, 1977). We proposed that this antigen may be derived from the merozoite surface coat. The surface coat has antiphagocytic properties (Brooks and Kreier, 1978) and antibody to it should aid the host to contain the infection. If freeze-thawing or washing dislodges only some of the antigenic components from the membrane, then antigens capable of stimulating an immune response may in fact be membrane associated as suggested by Speer et al., (1976) and still appear in the soluble components of the parasites.

Immunization studies must be combined with fractionation procedures and biochemical quantitation to determine if particular fractions of the parasite are enriched in antigens stimulating the protective response. In the work described in this report evidence derived from immunization studies and protein analysis is provided which shows that a soluble antigen obtained by washing sonically freed plasmodia is rich in those fractions of the parasite needed to stimulate a protective immune response in the host.

MATERIALS AND METHODS

Parasites

The *Plasmodium berghei* (WR) strain used in this study was obtained from Dr. M. Aikawa (Case Western Reserve University, Cleveland, Ohio). This strain, which originated from the Walter Reed Army institute of Research is highly pathogenic to mice and causes death, usually within a week of the onset of patency. Weanling rats may die when infected with this strain but adult rats usually survive.

A pool of parasitized mouse erythrocytes in Alsever's solution plus 10% (v/v) glycerol was stored in liquid nitrogen (-193°C). This method of storage was chosen instead of serial passage in mice to reduce the possibility of antigenic drift in the parasite reference strain.

Animals

Out bred Swiss Albino mice were used to recover parasites from liquid nitrogen storage. The parasites were given intraperitoneally after the inoculum had been allowed to thaw in tepid water. Similar mice were used for testing the infectivity of freeze-thawed and cryo-impacted preparations.

Adult Sprague Dawley rats (Fisher 344, Charles River animal labs, N. Wilmington, Mass.) were used as the source of free parasites on which protein determinations and polyacrylamide gel experiments were done. Inbred male CDF rats were used in all vaccination experiments, for antigen production, as the source of challange inocula and as the test animals. The vaccination experiments were carried out in young adult male rats weighing between 70 and 90 grams. The animals were alloted to the various groups randomly.

Harvesting of Free Parasites

Blood from infected rats (approximately 30-50% parasitemia) was drawn by

cardiac puncture into cold Alsever's solution. In order to attain this high *parasitemia*, the reticulocyte count was increased by injections of phenylhydrazine hydrochloride (1.5% aqueous solution). A dose of 30 mg/kg body weight was given on day 0 and day 2 of the harvest (Kreier et al., 1976).

The red blood cells were washed twice in Alsever's solution by centrifugation at 650g for 10 minutes and diluted to yield a 10% suspension of red blood cells. The suspension was passed through a column of Whatman #1 powdered filter paper packed lightly in a 50ml syringe to remove leukocytes. The erythrocytes were disrupted by treatment in a continuous flow sonication system (29.6 ml/minute; 20 kHz) by methods previously described (Prior and Kreier, 1972, 1972a).

The sonicated suspension was passed through a column containing glass wool to remove as many strands of DNA as possible. The suspension was then spun at 650g for 10 minutes to remove unbroken erythrocytes, fragments of erythrocytes and large free parasites. The supernatent, containing predominantly small free parasites, was collected and centrifuged at 6000g for 10 minutes yielding a brown pellet of parasites. The pellet was resuspended in physiological saline (0.15M NaCl) and washed once by centrifugation for 10 minutes at 6000g (Hamburger and Kreier, 1975; Kreier et al., 1976).

Freeze-thawing

Suspensions of free parasites in physiological saline were distributed in 8 x 75 mm test tubes. The test tubes were immersed in a dry ice-acetone bath until the parasite suspension was frozen solid. The test tubes containing the frozen parasites were then swirled in a 37°C water bath until there were no ice crystals visible in the suspension. This procedure was repeated for each cycle of freeze-thawing.

Cryo-impacting

Free parasites in physiological saline were frozen by placing the suspension directly in the liquid nitrogen filled cryo-impacting chamber. The cryo-impacting apparatus consists of a two piece stainless steel chamber containing a large steel ball which is free to move about within the confines of the chamber. The chamber and the ball are both chilled in liquid nitrogen prior to use. After additional liquid nitrogen and the material to be cryo-impacted have been added, the chamber is closed and fastened to a piston on an electric motor. When the engine is turned on, the up and down motion of the chamber causes the steel ball to beat against the walls of the chamber and crush the frozen sample. After the sample has been "cryo-impacted" for the desired amount of time, the chamber is opened and the frozen sample material is collected as a fine powder by scraping it from the sides of the chamber and the ball (Smucker and Pfister 1975).

Protein Determinations

Suspensions of free parasites were counted in a haemocytometer and resuspended in physiological saline for protein determination.

Cryo-impacted, freeze-thawed and control samples were centrifuged at 30,000g for 30 minutes and the pellet (insoluble) and the supernatent (soluble) fractions were both resuspended to the original volume. This procedure makes the soluble and insoluble components of the parasites in the fractions comparable to their concentrations in the original suspension.

We found it technically difficult to manipulate the cryo-impacted material in some experiments so the following microtechnique was designed: The cryo-impacted material was allowed to thaw in petri dishes at room temperature. The syrupy material was mixed with an applicator stick until it was homogenous.

The liquid was drawn into a capillary tube which was then sealed at one end. The soluble and insoluble fractions were separated, when applicable by centrifugation.

The inner diameter of the hematocrit tube was 0.55 mm. A 50 mm length of the tube filled with parasite material therefore contained a volume of 0.0475 ml. A 50 mm section of the tube containing the parasite material was cut and immersed in approximately 0.3025 ml of physiological saline and the parasite material was rinsed free of the tube. This procedure effectively produced a 1:8 dilution of the material which was easy to manipulate and suitable for the protein procedures.

Protein assays on all samples were performed according to the Lowry method (Lowry et al., 1951).

Packed Volume Determinations

After treatment, hematocrit tubes were used to collect samples of the parasite suspensions. The tubes containing the suspensions were centrifuged for 5 minutes at 12,000 g in an International Micro-Capillary Centrifuge. The packed volume of a control sample, which had not been treated, was arbitrarily designated as 100% packed volume. The packed volumes of all of the treated samples were compared to the packed volume of the control sample and the percent reduction of insoluble parasite material was calculated.

Electron Microscopy

Specimens for electron microscopy were prepared as previously described by Aikawa et al. (1968). Thin sectioning was done on a Reichert thermal advance microtome. Sections were stained by immersion in a drop of 2% uranyl acetate, rinsed and stained with lead citrate. Photographs were taken on the Hitachi HS-8 Electron Microscope.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE analysis was carried out on the various soluble fractions by methods previously described (Garvey et al., 1977). About 20 μg of a sample was added to each gel column and electrophoresis was carried out at 3-5 Ma for about 2 hours or until the Bromphenol blue tracking dye approached the bottom of the column. Gels were stained for protein with Coomassie Blue according to the procedure of Garvey et al. (1977) and destained by diffusion. Relative mobilities were calculated and compared.

Washoff Antigen

The washoff antigen was prepared by suspending about 4×10^9 whole free parasites in 2 ml cold physiological saline. The free parasites were spun gently with a magnetic stir bar and plate in the cold for 2 hours. The washed free parasites were then separated from the released material by centrifuging at 30,000g for 30 minutes. A dose of antigen was that amount of material contained in 0.25 ml of wash water.

Vaccinations

All vaccinations were done in 70 to 90 gram male CDF rats. Vaccine doses were 5×10^8 free parasites or a fraction of 5×10^8 free parasites. Vaccination was by intramuscular injection of the antigen with saponin (0.3125 mg) adjuvant. Challange was by intravenous injection of 1×10^4 parasitized erythrocytes. A detailed description of the vaccination procedure has been published (Saul and Kreier, 1977).

RESULTS

Effect of Multiple Cycles of Freeze-thawing on the Amount of Protein Solubilized and the Packed Volume of Residual Insoluble Material

In Table 1 the relationship between the number of cycles of freezing and thawing to which the parasites are subjected, the amount of protein solubilized and the amount of residual solids is shown. It is apparent that two cycles of freezing and thawing release as much protein as will be released by this procedure. Additional cycles of freezing and thawing in fact appear to decrease the amount of protein recoverable in the soluble fraction probably because some of the previously solubilized protein is denatured. The volume into which the residual material can be packed continues to decrease with additional cycles of freezing and thawing. The insoluble material remaining after 3 cycles of freezing and thawing consists of membrane fragments, membrane vesicles and amorphous debris (Figure 1).

Effect of Time of Cryo-impacting on Amount of Protein Solubilized and Packed Volume of Residual Insoluble Material

The relationship between duration of cryo-impacting, the amount of protein solubilized and the volume of residual insoluble material is similar to that which occurs with freezing and thawing. The amount of protein recoverable in the soluble fraction actually decreases slightly with increasing time of treatment (Table 2). The insoluble material obtained by a relatively short period of cryo-impacting (30 seconds) consists of membrane fragments, membrane vesicles and amorphous debris similar to that seen following freeze-thawing (figure 2).

The Number and Condition of Free Parasites Before and After Washing in Cold Saline for 2 Hours

Giemsa stains were made of free parasites before and after washing. By

Table 1. Effect of multiple cycles of freeze-thawing on the amount of protein solubilized and the packed volume of residual insoluble material

Number of cycles of freeze-thawing	% protein solubilized		% original packed volume	
	Ave ± SD	# Samples	Ave ± SD	# Samples
0	5 ± 2%	15	100%	8
1	28 ± 4%	8	91 ± 3%	4
2	34 ± 3%	8	81 ± 6%	4
3	30 ± 8%	12	58 ± 12%	6
4	29 ± 1%	6		
5	32 ± 6%	4		
6	30 ± 6%	9	49 ± 5%	6
7	31 ± 0%	4		
9	30 ± 9%	9	47 ± 1%	4
12	27 ± 2%	6	43 ± 3%	2
15	23 ± 9%	4		
21	26 ± 3%	3		

Figure 1. Electron micrograph of a preparation of free Plasmodium berghei which had been freeze-thawed three times. Membrane fragments (MF), a membrane bound vesicle (MV), and amorphous debris are visible (140,000X).

Table 2. Effect of time of cryo-impacting on the amount of protein solubilized and the packed volume of residual insoluble material

number of seconds cryo-impacted	% protein solubilized		% original packed volume	
	Ave ± SD	# Samples	Ave ± SD	# Samples
0	5 ± 2%	8	100%	4
LN only	43 ± 17%	4		
5	41 ± 10%	4		
15	46 ± 14%	6		
30	43 ± 11%	6	62 ± 8%	4
60	38 ± 5%	6	53 ± 10%	3
120	38 ± 5%	4	44 ± 1%	2
240	34 ± 2%	4	32 ± 2%	2

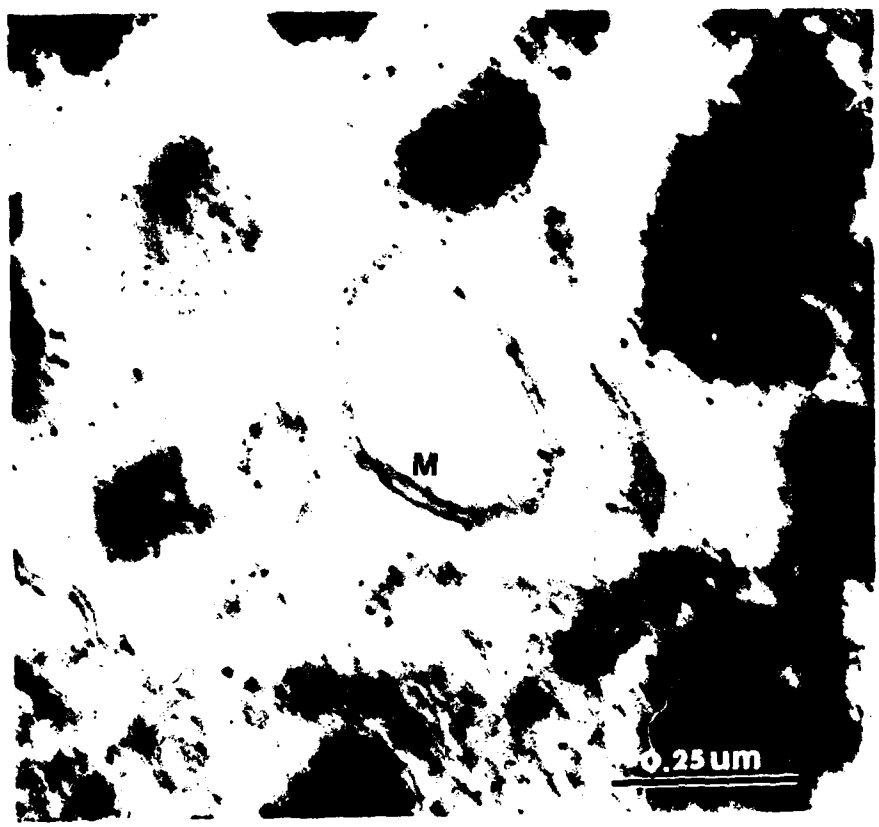

Figure 2. Electron micrograph of a preparation of free Plasmodium berghei which had been cryo-impacted for 30 seconds. Membrane fragments (M), and amorphous debris are visible (110,000X).

this technique no noticeable morphological deterioration in the washed parasites was detected. The numbers of parasites remaining in the suspensions after washing were determined by counting in a hemocytometer. The protein content in the suspension and in the wash water was also determined. The numbers of intact parasites recovered from the suspension after 2 hours of washing were not significantly different from the numbers present at the start of washing. About 4.5% of the parasites protein was released by the washing procedure. The data are presented in Table 3.

Patterns Obtained by Polyacrylamide gel Electrophoresis (PAGE) of Soluble Proteins from Free Plasmodium berghei

The patterns obtained by disc gel electrophoresis of the soluble materials released by freeze-thawing free parasites 3 times or by 30 seconds of cryo-impacting differed one from the other only slightly.

The bulk of the soluble material washed from the parasites resolved into several bands (i.e. 2 to 4) with migration coefficients of 45 to 55. Bands were present in the same region in the disc gels of the soluble material from the freeze-thawed and cryo-impacted parasites (Table 4).

Rat haemoglobin resolved into four bands with migration coefficients of 22, 29, 35, 43.

Immunization Studies

In the immunization studies 70 to 90 gram CDF male rats were given one injection of 5×10^8 parasites (merozoites and small trophozoites) or a fraction containing a component of 5×10^8 free parasites. Control rats received a volume of red cell membranes equal to the volume of 5×10^8 free parasites. The protein content of our various antigen preparations differed depending on the fraction of the parasite represented by that antigen. An average small free parasite, i.e. merozoite or small trophozoite, contained about 2.71 ± 0.65 x

Table 3. The number and condition of free parasites before and after washing in cold saline for 2 hours

Parasites/ml		Protein in Parasites	Protein in "Wash Off" Antigen	
Before Washing	After Washing	(µg/ml)	(µg/ml)	%
2×10^9	1.86×10^9	5804	350	6.0
2×10^9	2.07×10^9	6625	217	3.2
2×10^9	1.95×10^9	4721	200	4.2
Average	1.96×10^9	5717	255	4.5%

Table 4. Migration coefficients of soluble antigens released by washing, freeze-thawing (FT) or cryo-impacting (CI) free *P. berghei*.

Wash Off	Free Parasites F-T	C-I	Rat Hemoglobin
	12	12	
	18	18	
			22
	24		
		25	
	29		29
		30	
	33	33	
			35
	38	38	
	41	41	
			43
45			
↑	49		
		50	
↓			
55	55	55	
	61	61	
	73	73	
	79	79	
	85		
		88	

*
These figures are calculated from 4 separate runs for the "wash off antigen", 8 runs each for the F-T and C-I material, and 2 runs for rat hemoglobin.

10^{-6} µg of protein (Table 5). Reasonably carefully handled parasites lost about 6% of their protein into solution. Freeze-thawing three times released about 35% of the protein from the parasites (Table 6). One vaccine dose of 5×10^8 free parasites contained 1294±147 µg of protein. The soluble fraction of 5×10^8 freeze-thawed parasites contained 472±98 µg of protein; the insoluble fraction 944±118 µg. The respective figures for cryo-impacted parasite vaccines were 562±105 µg in the soluble and 750±55 µg in the insoluble fraction. Seventy ± 20 µg of protein were present in each dose of "wash off" antigen (Table 7).

Of the various vaccine preparations tested a single dose of 5×10^8 free parasites gave the best protection. The protective antigens partitioned between the soluble and insoluble fractions on freeze-thawing or cryo-impacting (Figures 3 and 4). Five $\times 10^8$ washed free parasites were less potent than 5×10^8 unwashed parasites and the material washed from 5×10^8 free parasites was about as immunogenic as were the washed parasites (Figure 5). This despite the fact that the "wash off" antigen contained only about 4.5% (Table 3) of the protein in the parasites.

Table 5. Protein content (μg) calculated for an average free small parasite in a mixed population of small free Plasmodium berghei.

PARASITES/ML	PROTEIN(μg/ml)	UG/PARASITE
1×10^9	2290	2.29×10^{-6}
1×10^9	2431	2.43×10^{-6}
2×10^9	5804	2.90×10^{-6}
2×10^9	6625	2.65×10^{-6}
5×10^8	1080	2.16×10^{-6}
5×10^8	1136	2.31×10^{-6}
5×10^8	2096	4.20×10^{-6}
AVERAGE		$2.71 \pm 0.65 \times 10^{-6}$

Table 6. Protein content (µg/ml) of soluble and insoluble components of three times freeze-thawed small *Plasmodium berghei* free parasites. Percent of total protein is in parenthesis.

parasites per ml	Before FT		After FT	
	total prot.	sol prot.	sol prot.	insol. prot.
5×10^8	1240	40(03%)	400(32%)	1120(90%)
5×10^8	1144	60(05%)	412(36%)	824(72%)
1×10^9	2560	316(12%)	1001(39%)	1997(78%)
1×10^9	2681	99(04%)	831(31%)	2014(75%)
2×10^9	6240	426(07%)	2536(41%)	3584(57%)
2×10^9	4800	321(07%)	1516(32%)	3280(68%)
AVERAGE		6% ± 3%	35% ± 4%	73% ± 11%

Table 7. Protein concentrations in various types of vaccine. All vaccines contained 5×10^8 free parasites or a fraction derived from 5×10^8 free parasites in 0.25 ml of saline.

	Protein (µg)	
	Ave + SD	# determinations
5×10^8 Free parasites	1294 ± 147	6
Sol. fract.(F-T) from 5×10^8 free parasites	472 ± 98	6
Insol. fract. (F-T) from 5×10^8 free parasites	944 ± 118	6
Sol. fract.(C-I) from 5×10^8 free parasites	562 ± 105	3
Insol. fract. (C-I) from 5×10^8 free parasites	750 ± 55	3
Wash off from 5×10^8 free parasites	70 ± 20	4

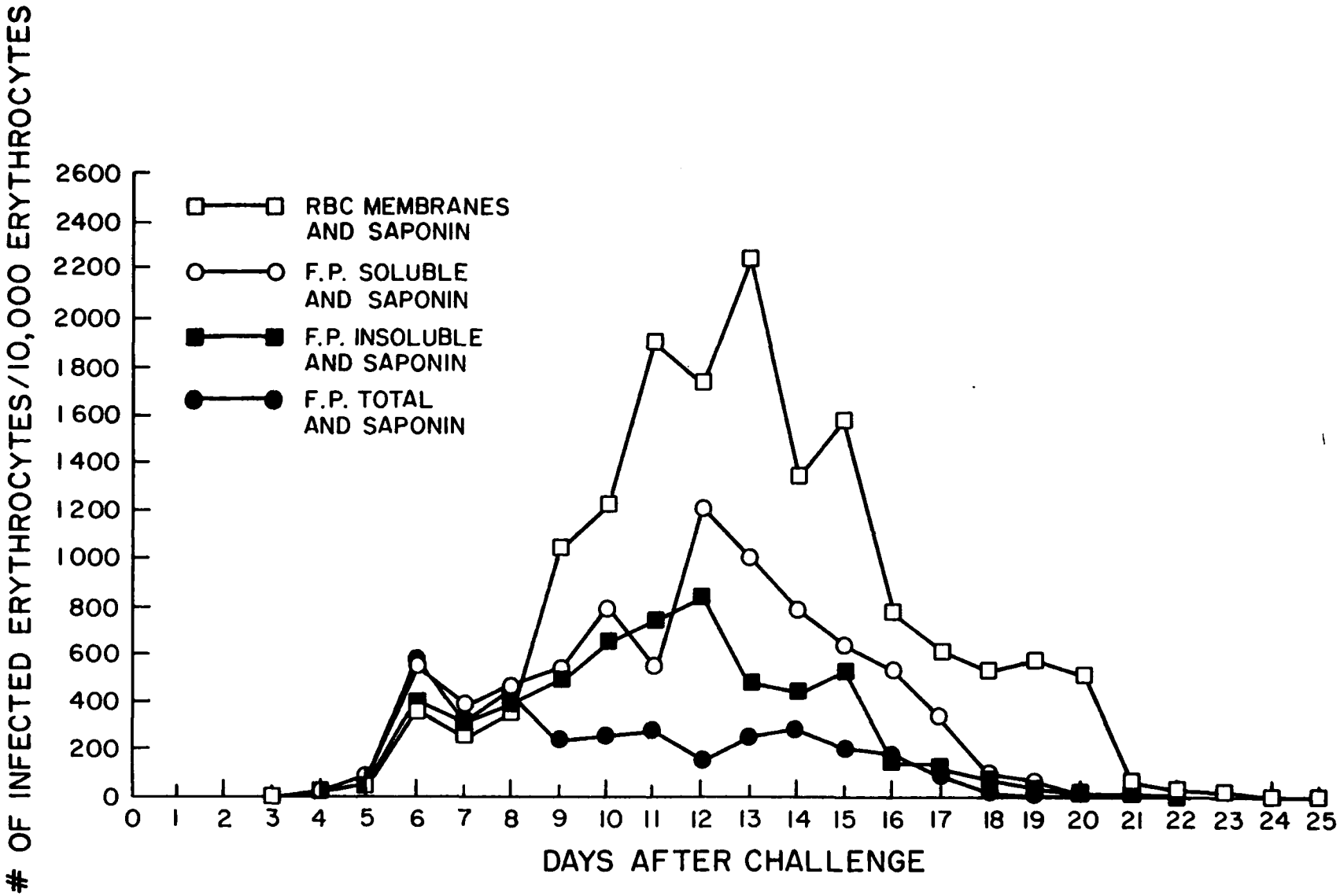

Figure 3. Parasitemias following challenge in rats given various preparations of freeze-thawed free Plasmodium berghei. Vaccination with 5×10^8 free freeze-thawed parasites gave the greatest protection. The soluble and insoluble fractions also stimulated good protection but somewhat less than did the unfractioned parasites. The dose of unfractioned parasites contained 1294 ± 147 µg of protein, the soluble fraction 472 ± 98 µg. Each point is an average derived from the 4 rats in each group. A duplicate experiment (data not shown) gave similar results.

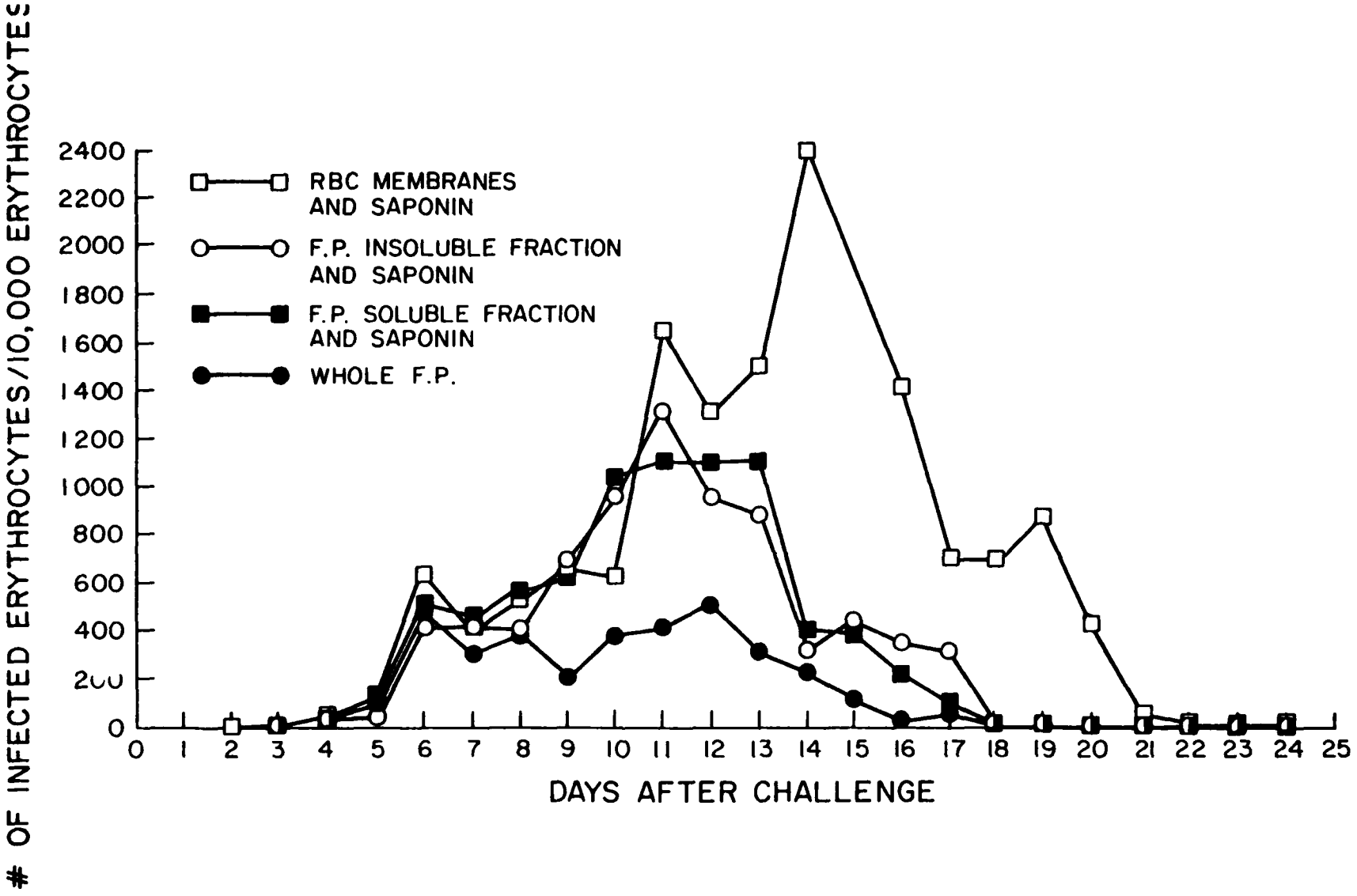

Figure 4. Parasitemias following challenge in rats given various preparations of cryo-impacted free Plasmodium berghei. Vaccination with 5×10^8 free cryo-impacted free parasites gave the greatest protection. The soluble and insoluble fractions also gave good protection, but somewhat less than did the infractionated parasites. The dose of unfractioned free parasites contained 1294 ± 147 µg of protein, the insoluble fraction 750 ± 55 µg and the soluble fraction 562 ± 105 µg. Each point is an average derived from the 4 rats in each group. A duplicate experiment (data not shown) gave similar results.

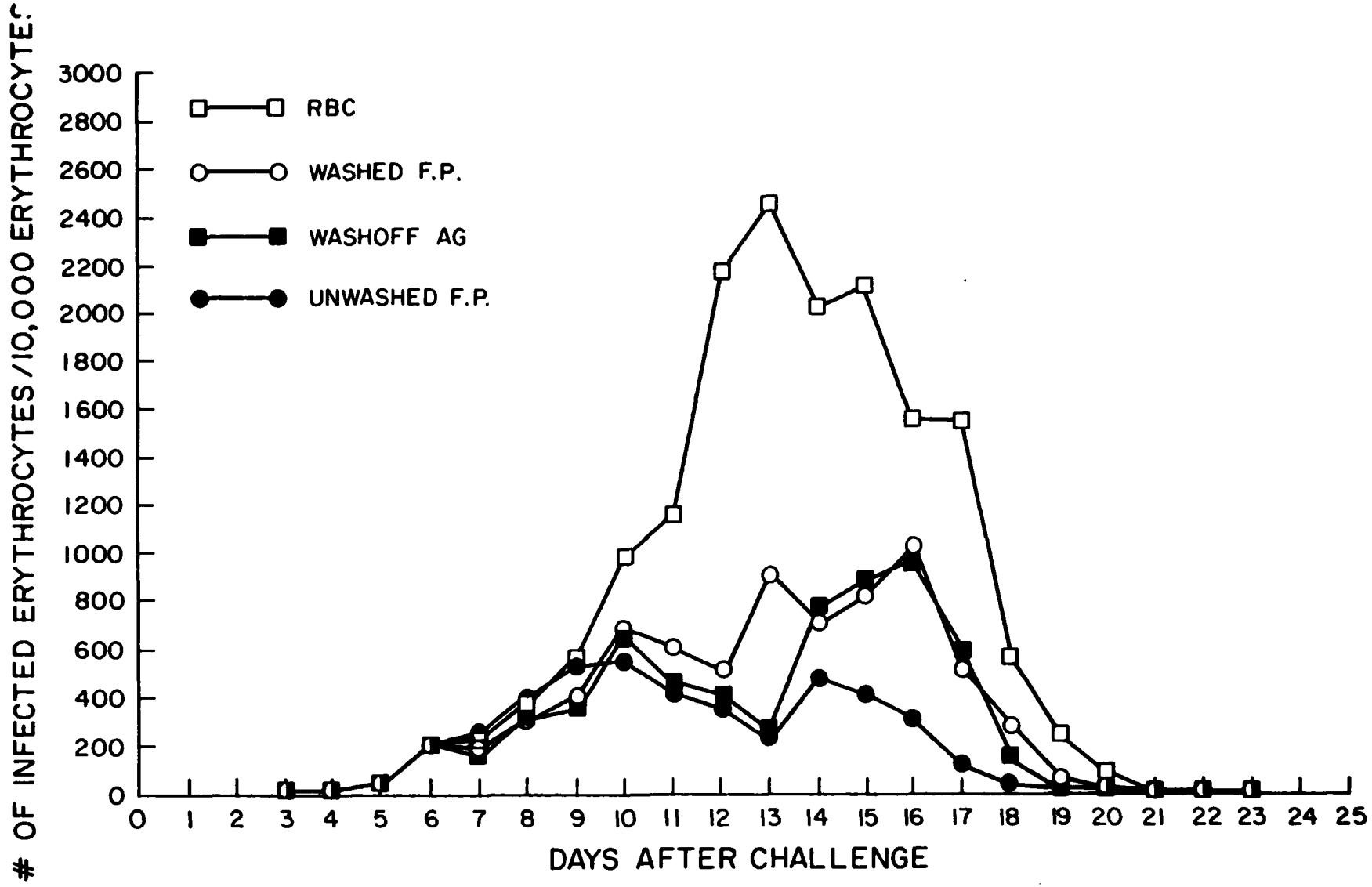

Figure 5. Parasitemias following challenge in rats given various preparations of free Plasmodium berghei. The unwashed free parasites gave the best protection. The washed parasites and the washings from the parasites gave slightly less strong protection but still fairly strong protection. One dose of the washed and unwashed parasites contained 129 ± 147 µg protein; the dose of "washoff" antigen contained 70 ± 20 µg of protein and on polyacrylamide gel electrophoresis resolved into only 2 to 4 bands of similar relative mobility. Each point is an average derived from the four rats in the group. A duplicate experiment (data not shown) gave similar results.

DISCUSSION

In earlier work, (Saul and Kreier 1977) we observed that an immunogen could be obtained by gentle washing of sonically released parasites. In the present study, we confirmed that observation and obtained more information about the characteristics of this material. Soluble material washed from the surfaces of free parasites was fractionated by analytical electrophoresis on polyacrylamide gel. This material was compared with the soluble portion of 30 second cryo-impacted and three times freeze-thawed parasites. The materials released by the cryo-impacting and freeze-thawing procedures were very similar. Material obtained by both procedures yielded band patterns similar in density and distribution on polyacrylamide gel electrophoresis (Table 4). Approximately 13 bands of protein were obtained on these gels. These results are similar to those of others who obtained 8 to 16 bands by PAGE fractionation of soluble extracts of Plasmodium berghei (Chavin, 1966; Spira and Zuckerman, 1966; Sodeman and Meuwissen, 1966; Corradette et al., 1971; Hamburger and Zuckerman, 1976; 1976a; Kreier et al., 1976).

On the other hand, soluble material washed from the parasites resolved into only 2-4 major bands on the gels. Most of the bands in the gels of the freeze-thawed or cryo-impacted parasites could be detected in the gels of the "washoff" antigen, but they were very faint. This is not unexpected as some plasmodia certainly lysed during the washing. The major difference between the "washoff" preparations and the freeze-thawed preparation is in the relative concentration of the constituents. The gels are always loaded with approximately 20 μg of material, so if the amount of one component in the preparation is larger, then that of the others must be smaller.

The results of polyacrylamide gel electrophoresis of soluble antigens washed from parasites obtained in this study differ somewhat from those reported in our earlier study. In the earlier study (Saul and Kreier 1977) the material in the "washoff" antigen concentrated primarily in one thick band with a relative mobility of between 56 and 60. In the present, more extensive studies, the "washoff" antigen resolved into 2 to 4 bands with relative mobilities between 45 and 55. In the earlier work protein concentrations were not as carefully controlled as they were in the present studies and this as well as other minor differences in technique may account for the discrepancy.

The sonically freed parasites from which we obtained our antigens are released in cold saline and promptly collected by centrifugation. The immunogenic material is released easily by gentle agitation in cold saline, but some of the protection stimulating antigen is tightly bound on the parasites membranes also as it remains there after 3 cycles of freeze-thawing. It is probable that similar gentle washing of parasites released by some other means would not yield the antigen we obtain from our sonically released parasites as it would probably be lost during the manipulations involved in obtaining the parasites. Parasites released naturally in culture, may for instance, remain in warm culture medium for some time before they can be collected and if collected by seiving they may be exposed to very large volumes of culture medium. Under these conditions any loosely attached surface antigens would probably be lost.

M. Ristic of the University of Illinois has recently informed us by personal communication that he has obtained a "washoff" antigen from free Babesia bovis merozoites. This antigen like our "washoff" antigen contains only a few of the proteins present in the merozoites but nonetheless stimulates development of immunity to B. bovis in cattle.

We determined that our small free parasites contain approximately 2.7 x 10^{-6} µg of protein. It must be taken into consideration that the various populations of parasites analyzed in these experiments did not always contain the same proportions of the various morphological stages of the parasites. Although the procedure used to obtain parasites selected for a population of small free blood stage parasites (Kreier et al., 1976), actual numbers of small trophozoites and merozoites may have varied depending on the state of infection in the donor host at the time of bleeding. Trophozoites have a large size range, therefore, even if the number of parasites tested was exactly as reported, variations in the proportions of trophozoites and merozoites could cause considerable variations in the amount of protein per parasite.

The results of subjecting parasites to between 0-21 cycles of freeze-thawing indicate that the amount of protein solubilized by one or two cycles of freeze-thawing was comparable to the amount of protein which was solubilized by 21 cycles of freeze-thawing (Table 1). These data indicate that freeze-thawing is capable of lysing the parasites and releasing the internal components, but little additional release of protein bound in organelles and membranes is accomplished by increasing the number of freeze-thawings. These results are similar to results reported by Lunde and Powers (1976). In order to determine if additional antigen could be obtained by more than one cycle of freeze-thawing,

Lunde and Powers (1976) freeze-thawed a population of parasites four additional times after an initial treatment. They reported that only a small additional amount of antigen was obtained by the repeated cycles of freeze-thawing. Dulaney and Morrison (1944), in contrast, found that one cycle of freeze-thawing released only a small amount of the soluble material. They reported that four additional cycles of freeze-thawing yielded "1/4 to 1/2 the activity of the first cycle". These results are probably not directly comparable to ours as both of these groups were measuring antigenic activity rather than release of a chemically defined substance. We concluded that one cycle of freeze-thawing is probably sufficient to release most of the soluble protein which it is possible to release by this procedure, but that three cycles of freeze-thawing would assure maximum release.

Cryo-impacting released a maximum of about 40% of the total protein contained in the parasites (Table 2). Five seconds of cryo-impacting apparently solubilized as much protein as 240 seconds of cryo-impacting. The sample which was simply frozen in liquid nitrogen in the cryo-impacting chamber and removed before the machine was turned on was included to determine the amount of protein released during the one cycle of freeze-thawing inherent in the cryo-impacting procedure. It is significant that the mechanical action released very little more protein from the cryo-impacted samples than did the freeze-thawing in liquid nitrogen. The decision to compare cryo-impacting with freeze-thawing by measuring the amount of protein solubilized was arbitrary. Other components of the parasite including RNA, DNA, and lipids, are also present in the parasites and will be studied in future work.

By measuring the packed volume of the treated parasite preparations, it was possible to make a rough comparison of the physical size of the insoluble fragments after treatment. The volume into which the insoluble residue of the

freeze-thawed (Table 1) and the cryo-impacted samples (Table 2) could be packed decreased with increases in the amount of treatment. This suggests that the large insoluble fragments present after initial lysis are broken into smaller pieces which pack more tightly as treatment continues.

Electron micrographs of a selected population of untreated Plasmodium berghei free parasites have been published (Kreier et al., 1976). These preparations consist of a mixture of merozoites and small trophozoites. The parasites appear to morphologically intact and surrounded by a complete membrane. The electron micrographs of the freeze-thawed (Figure 1) and cryo-impacted (Figure 2) parasites show insoluble malarial pigment (hemozoin), membrane bound vesicles, and fragmented parasite membranes. Both procedures disrupt the parasite membranes and release the cytoplasmic contents of the cell. Although both treatments lysed a very high percentage of the parasites, occasional unbroken and apparently undamaged parasites were observed even in extensively treated preparations. These observations were confirmed by the results of related studies which showed that enough viable parasites were present in all cryo-impacted and freeze-thawed preparations to initiate infections in mice.

The "washoff" antigen and the washed parasites as well as the soluble and insoluble fractions of freeze-thawed and cryo-impacted free parasites were compared in the standardized system for evaluating the immunogenicity of Plasmodium berghei in rats developed earlier in this laboratory (Saul and Kreier, 1977). In the current studies the actual amount of protein in each dose of antigen was determined (Table 7).

The protection stimulating antigen partitioned between the soluble and insoluble fractions of three times freeze-thawed and 30 second cryo-impacted free parasites. The soluble portions in both cases, however, contained somewhat less

total protein than the insoluble. As these two preparations were roughly equivalent in their immunogenicity this implies that the soluble portion may be slightly richer in protective antigens. The immunogenicity of vaccines prepared by cryo-impacting or freeze-thawing, appeared to be similar.

The results of vaccination with the soluble material washed from 5×10^8 free parasites (washoff antigen) and with 5×10^8 washed free parasites are shown in Figure 5. Although neither the washoff antigen nor the washed free parasites induced as strong protection as did the unwashed free parasites, they both induced a significant degree of protection. It is an important point that the amount of protein in the dose of washoff antigen (70 ± 20 µg) was much less than the amount of protein in the dose of washed or unwashed free parasites (1294 ± 147 µg). The fact that an antigen fraction containing only 1/20 the parasites protein stimulates almost as strong immunity as does the whole parasite suggests that the washoff material must be a concentrated preparation of the protection stimulating immunogen. This supposition is supported by the polyacrylamide analyses which show that the "washoff" antigen resolves into only a few bands (2 to 4) while soluble extracts of the parasite resolve into 13 bands.

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